Department of Ophthalmology
University of Helsinki
Helsinki, Finland

ACUTE ANTERIOR UVEITIS AND HLA-B27:

INFECTIOUS BACKGROUND,

SYSTEMIC INFLAMMATION,

AND PROGNOSIS OF THE PATIENTS

Minna Huhtinen

Academic dissertation

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Supervised by:

Docent Anni Karma, MD
Department of Ophthalmology
University of Helsinki, Finland

and

Professor Marjatta Leirisalo-Repo, MD
Department of Internal Medicine
Division of Rheumatology
University of Helsinki, Finland

Reviewed by:

Professor T.E.W. Feltkamp, MD
University of Amsterdam
Amsterdam, The Netherlands

and

Docent Marianne Gripenberg-Gahmberg, MD
Tammisaari Regional Hospital
Tammisaari, Finland

Discussed with:

Professor (emer.) Kimmo Aho, MD
National Public Health Institute, Helsinki, Finland

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ABBREVIATIONS

AAU	acute anterior uveitis
ABC	antibody binding capacity
AS	ankylosing spondylitis
AU	anterior uveitis
BSA	bovine serum albumin
CD	cluster of differentiation, classification system for outer membrane structures of cells, mostly glycoproteins
C. jejuni	*Campylobacter jejuni*
CME	Cystoid macular edema
Cpn	*Chlamydia pneumoniae*
Ctr	*Chlamydia trachomatis*
CRP	C-reactive protein
CU	colitis ulcerosa
E. coli	*Escherichia coli*
EIU	endotoxin induced uveitis
ELISA	enzyme-linked immunosorbent assay
EMIU	experimental melanin-induced uveitis
FACs	fluoresence activated cell sorter
FasL	Fas ligand
HLA-B27	human leukocyte antigen B27
Hsp60	heat shock protein 60
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IL	interleukin
IL-1RA	IL-1 receptor antagonist
IOP	intraocular pressure
K. pneumoniae	*Klebsiella pneumoniae*
LFA-1	lymphocyte function-associated molecule 1
LPS	lipopolysaccharide, endotoxin
LTB_4	leukotriene B_4
MHC	Major Histocompatibility Complex

MIF	microimmunofluorescence
mRNA	messenger ribonuclein acid
NO	nitric oxide
NOS	nitric oxide synthase
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
P. mirabilis	*Proteus mirabilis*
ReA	reactive arthritis
S. enteritis	*Salmonella enteritis*
sIL-2R	soluble interleukin-2 receptor
S. typhimurium	*Salmonella typhimurium*
SpA	spondyloarthropathy
TCR	T cell receptor
TGF-β	transforming growth factor β
Th	T helper
TNF-α	tumor necrosis factor alpha
UC	ulcerative colitis
Y. enterocolitica	*Yersinia enterocolitica*
Y. pseudotuberculosis	*Yersinia pseudotuberculosis*

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by the Roman numerals **I** to **IV**.

I Huhtinen M, Karma A. HLA-B27 typing in the categorization of uveitis in a HLA-B27 rich population. *Br J Ophthalmol* 2000;84:413-416.

II Huhtinen M, Laasila K, Granfors K, Puolakkainen M, Seppälä I, Laasonen L, Repo H, Karma A, Leirisalo-Repo M. Infectious backround of patients with a history of acute anterior uveitis. *Ann Rheum Dis* 2002;61:1012-1016.

III Huhtinen M, Puolakkainen M, Laasila K, Sarvas M, Karma A, Leirisalo-Repo M. Chlamydial antibodies in patients with previous acute anterior uveitis. *Invest Ophthalmol Vis Sci* 2001;42:1816-1819.

IV Huhtinen M, Repo H, Laasila K, Jansson S-E, Kautiainen H, Karma A, Leirisalo-Repo M. Systemic inflammation and innate immune response in patients with previous anterior uveitis. *Br J Ophthalmol* 2002;86:412-417.

1. ABSTRACT

The aim of the present study was to increase our knowledge of the use of HLA-B27 typing in the diagnostic work-up of uveitis in a HLA-B27 rich population, the clinical picture and outcome of patients with HLA-B27 positive and negative unilateral acute anterior uveitis (AAU), and further, to explore the infectious background, systemic inflammation and innate immune responsiveness of patients with previous AAU.

Between 1993 and 1996, 220 consecutive patients with undetermined uveitis at onset were examined in the Helsinki University Eye Hospital. HLA-B27 antigen was tested in 85% of the patients. Other laboratory or *x* ray examinations were performed on the basis of the anatomical classification of uveitis and the biomicroscopic features characteristic of uveitis associated with systemic diseases.

HLA-B27 antigen was found significantly more often in patients with anterior (71%) uveitis than in patients with intermediate, posterior, or panuveitis (7%). Further, compared with acute or recurrent unilateral (79%) forms, HLA-B27 antigen was rare in chronic (7%) or bilateral (12%) forms. Of the 16 cases of HLA-B27 negative unilateral AAU, five showed biomicroscopic features representing uveitis entities. The remaining 11 cases did not differ in any respect from the cases of HLA-B27 positive unilateral AAU.

The results indicate that the determination of HLA-B27 antigen helps the clinician in the diagnostic work-up of unilateral AAU. Positive test results serve as a clue to search for spondyloarthropathies, and negative results indicate the need to look for specific uveitis entities and other systemic diseases. The occurrence of HLA-B27 positivity in conjunction with uveitis entities other than unilateral AAU is of the same level or less than in the population of Finland in general.

In 1999 altogether 64 patients with previous AAU were examined in a follow-up visit and blood samples were taken from the patients and 64 sex- and age-matched controls. Serum antibodies to *Salmonellae, Yersiniae, Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Campylobacter jejuni,* and *Borrelia burgdorferi* were measured using enzyme-linked immunosorbent assay (ELISA),

serum antibodies to *Chlamydia trachomatis* and *Chlamydia pneumoniae* (Cpn) by microimmunofluorescence test, and to *Chlamydia pneumoniae* heat shock protein 60 (Cpn Hsp60) by enzyme immunoassay (EIA). Peripheral blood mononuclear cells (PBMC), separated by density gradient centrifugation, were studied for *Salmonella* and *Yersinia* antigens using immunofluorescence test, and for *Chlamydia pneumoniae* DNA using polymerase chain reaction (PCR).

To determine innate immune responsiveness of patients with a history of AAU but no signs of ocular inflammation at the time of recruitment in comparison with healthy controls, tumor necrosis factor (TNF)-α production in response to bacterial lipopolysaccharide (LPS) was studied using whole blood culture assay. The levels of TNF-α in culture supernatants and soluble interleukin-2 receptor (sIL-2R) in serum were determined by chemiluminescent immunoassay (Immulite®). The monocyte surface expression of CD11b, CD14, and CD16 and the proportion of monocyte subsets $CD14^{bright}CD16^{-}$ and $CD14^{dim}CD16^{+}$ were analyzed by three-color whole blood flow cytometry. For the evaluation of systemic inflammation the serum C-reactive protein (CRP) levels were determined using immunonephelometric high-sensitivity CRP assay.

Neither prevalence nor levels of single microbial antibodies studied differed between the patients and control subjects, or between subgroups of patients created on basis of clinical characteristics. The levels of immunoglobulin (Ig) A antibodies to *Chlamydia pneumoniae* heat shock protein 60 (Cpn Hsp60) were significantly higher in the AAU patients than in the controls in contrast to the levels of IgG antibodies to Cpn Hsp60. In comparison between patients with presence or absence of IgA antibodies to Cpn Hsp60, ocular complications were observed more often in the former group. In logistic regression analysis, high number of recurrences (>10) of AAU was independently related to the presence of single or multiple bacterial antibodies. None of the PBMC samples of the patients were positive for Y*ersinia* or *Salmonella* antigens. *Chlamydia pneumoniae* PCR was positive in a patient who was negative for *Chlamydia pneumoniae* antibodies.

The CRP level was significantly higher in the 56 patients with previous AAU than in the 37 controls. The tumor necrosis factor alpha (TNF-α) concentration of culture media per 10^5 monocytes was significantly higher in the patient group than in the control group in the presence of LPS 10 ng/mL and LPS 1000 ng/mL. The basal TNF-α release into culture media was low in both groups. The CD14 expression of $CD14^{bright}CD16^{-}$ monocytes, defined as antibody binding capacity (ABC), was similar in the patients and controls.

Although neither the prevalence nor the levels of single microbial antibodies studied differed between the patients and the controls, our results suggest that the presence of single or multiple antibodies in patients with many recurrences of AAU compared with patients with none or few recurrences may be a sign of repeated infections, antigen persistence and/or elevated innate immune responsiveness.This is supported by the finding of the high frequency of IgA antibodies to Cpn Hsp60 in patients with past AAU, indicating that such patients may have persisting or recurrent infections due to *C. pneumoniae* and that *C. pneumoniae* may play a role in the pathogenesis of AAU. The elevated CRP observed suggests that low-grade inflammation occurs in patients with a history of AAU. Increased TNF-α production by LPS-stimulated blood denotes enhanced innate immune responsiveness and may play a role in the development of ocular inflammation.

2. INTRODUCTION

Acute anterior uveitis (AAU) of unknown etiology is an inflammatory disorder that occurs in the iris and/or anterior ciliary body and lasts no more than three months. AAU is the most common form of uveitis and accounts for approximately three fourths of cases with annual incidence rate of about 8 cases per population of 100,000. Redness, pain, and photophobia are typical symptoms of which patients are complaining. The major indicators of AAU are the presence of cells and flare in the anterior chamber. Anterior chamber inflammation is assessed on slit-lamp biomicroscopy and responds well to topical corticosteroid therapy. Although AAU is usually the most easily managed form of uveitis, associated complications such as glaucoma may result in severe visual loss (Nussenblatt et al., 1996).

AAU belongs to a spondyloarthropathy (SpA) family, a heterogeneous group of rheumatic disorders that have a number of features in common. In addition to uveitis the typical disorders belonging to the SpA group are ankylosing spondylitis (AS), Reiter's syndrome/reactive arthritis (ReA), arthritis in association with inflammatory bowel disease (IBD), and psoriatic arthritis. There is clinical evidence of overlap between the various SpAs and a tendency towards familial aggregation. SpAs are characterized by involvement of the sacroiliac joints, by peripheral inflammatory arthropathy, and insertional tendinitis (Calin 1998). Although there are still open questions about the etiopathology of SpA, it is considered to involve genetic factors like human leukocyte antigen B27 (HLA-B27) and environmental factors like infections (Rose, 1998). Over 50% of AAU patients have been reported to possess the HLA-B27 antigen (Brewerton et al., 1973a, Ehlers et al., 1974, Linssen et al., 1991). In the acute phase of the disease most patients with AAU do not have clinical infection and laboratory techniques have often failed to give evidence of infections associated with the disease (Sprenkels et al., 1996b). In contrast, in patients with ReA where an infection is a triggering event, presence of microbial antigens in the joint has been demonstrated (Gaston et al., 1999). In addition, higher incidence and levels of antibodies to causative bacteria has been detected in arthritic patients compared with non-arthritic controls (Aho et al.,1979). Further, persistence of microbial antigens has been shown for prolonged periods in circulation (Granfors et al., 1998), in the gut and in the skin (Hoogkamp-Korstanje et al., 1988). In ankylosing spondylitis (AS), a prototype of SpA, direct evidence of enhanced jejunal production of antibodies to Enterobacteria has been shown (Mäki-Ikola et al., 1997b).

However, little is known about the persistence of microbial antigens in patients with AAU and about factors leading to recurrent and/or complicated course of the disease in some of the patients. Moreover, the most fundamental question that arises is what sort of a role do systemic inflammation and innate and adaptive immune responses play in the pathogenesis of AAU.

3. REVIEW OF THE LITERATURE

3.1 Acute anterior uveitis

3.1.1 Epidemiology

AAU is one of the commonest uveitis entities diagnosed in both tertiary eye care centers and in general practices of ophthalmology accounting for two thirds of the uveitis cases (Smit et al., 1993, McCannel et al.,1996). The prevalence of AAU (a total number of active cases in the population at a given time) is approximately 1.1/1000 and in the HLA-B27-positive population 10/1000. An annual incidence of AAU has varied between 12 and 16 per 100,000 inhabitants (Vadot et al., 1984, Saari, 1984, Darrell et al, 1962). A lifetime cumulative incidence, indicating the number of people who have ever had definite AAU without known etiology, is approximately 2/1000 in Caucasian population, and 10/1000 in the HLA-B27-positive population (Linssen et al.,1991).

3.1.2 Clinical manifestations

AAU is unilateral in nature but can affect one eye after the other in a short period of time. Recurrences are common and in most cases one eye will be involved more than the other. The typical symptoms in patients with AAU are redness, pain, and photophobia. Tearing may occur and in severe cases patients complain of blurred vision (Nussenblatt et al., 1996).

On clinical examination ciliary flush, conjunctival injection in the perilimbal area, miosis and dilated iris vessels are common findings. Anterior chamber inflammation may vary from few cells and slightly observable flare by biomicroscopy to severe inflammation with fibrin clot, hypopyon, and anterior or posterior synechia formation. The cells and flare represent extravasated inflammatory cells and protein as a result of a breakdown of the blood-aqueous barrier. A hypopyon is composed of layered leukocytes and can occasionally be seen in anterior uveitis entities (Nussenblatt et al., 1996). In rare cases hyphema may also occur, but usually resolves without permanent damage (Fong et al., 1993). Inflammatory cells may also collect and adhere to the corneal endothelium and form small or medium sized so-called nongranulomatous keratic precipitates. Cellular reaction in the anterior vitreous may be

absent or marked, and may result in peripheral vitreous condensation simulating the ”snow bank” seen in intermediate uveitis (Nussenblatt et al, 1996).

In the acute phase of the inflammation, the intraocular pressure (IOP) can be decreased because of ciliary body shutdown with decreased aqueous production. As the inflammation subsides the intraocular pressure normalises but also may rapidly increase, especially in cases with severe synechia. Some patients are corticosteroid responders explaining the elevated IOP in some cases (Nussenblatt et al, 1996).

Acute or recurrent anterior uveitis (AU) may turn into chronic course needing continuous use of corticosteroids. In these cases the risk for complications is marked. Indeed, complications may be more sight-threatening than the inflammation itself. As indicated above, secondary glaucoma in the majority of cases of chronic AU is due to corticostreroid use (BenEzra et al., 1997). It may also be due to blockage of the trabecular meshwork by inflammatory cells or debris; inflammation of the trabecular meshwork; persistent peripheral anterior synechiae; posterior synechiae with iris bombé; forward rotation of the ciliary body and secondary angle closure; or in rare cases it may follow neovascularization of the angle and trabeculum area (Moorthy et al., 1997).

Posterior synechiae are frequently more extensive than suspected on clinical examination and may involve complete adhesion of much of the posterior iris surface to the lens. In the absence of glaucoma, posterior synechiae may produce a persistently small pupil and may affect visual acuity. In some cases the pupil may be very small with fibrin deposits filling the pupillary space, occluding it completely (seclusio pupillae) and markedly affecting the vision (Nussenblatt et al., 1996).

Cataract is one of the commonest complications in AU. It is observed in various degrees of severity in many cases of recurrent or chronic AU. In some patients cataract formation may be due to prolonged use of corticosteroids. In most cases, however, it is also associated with the inflammatory process and the release of cataractogenic cytokines (Hooper et al., 1990).

Chronic aqueous hyposecretion, hypotony, may result from chronic inflammation of the ciliary body, increased aqueous outflow through disrupted uveoscleral pathways, or cyclitic membrane formation and subsequent ciliary body and retinal detachments. Chronic hypotony can lead to degenerative changes in ocular tissues and eventual phtisis (Nussenblatt et al., 1996).

Cystoid macular edema (CME) occurs in cases of iridocyclitis or pure cyclitis but not in simple iritis. CME is usually, but not always, associated with decreased central visual acuity or metamorphosia, or

both. Most cases of CME occur because the chronic low grade inflammatory disease has not been recognized, has responded poorly to an optimal treatment regimen or has been undertreated. The earliest clinical signs of CME are a loss of the foveal reflex and a wet, glistening reflex in the posterior pole. In more severe cases cystoid accumulations of fluid surround the macula in a petaloid appearance. CME and optic disc edema may occur together when there is persistent hypotony. Left untreated, chronic changes may result in degeneration of photoreceptors, lamellar hole formation, and permanent decrease in central vision (Nussenblatt et al., 1996).

3.1.3 Predisposing factors

The majority of patients with AAU have no obvious precipitating event (Rosenbaum et al., 1991). A study of seasonal variation has been reported showing peak in the prevalence in the fall (Rothova et al.1987). Although the etiopathology of AAU and other forms of SpA is not known, it is considered to involve genetic and environmental factors, such as infections (Rose, 1998) or even trauma (Rosenbaum et al., 1991). More than 50 % of AAU patients are positive for the HLA-B27 antigen (Brewerton et al., 1973b). In Finland the figure is even higher, 80% (Saari, 1984). Prevalence of AAU in the HLA-B27-positive population is only 1%, (Linssen et al., 1991) but 13% of HLA-B27-positive first degree relatives of HLA-B27-positive patients suffer from AAU as well (Derhaag et al., 1988). A plausible explanation for this observation is that the disease has more than one genetic factor in addition to HLA-B27. AAU occurs in 5% of patients with acute ReA (Leirisalo et al., 1982). Among patients with AAU but no signs of ReA, microbes indicating an infectious etiology are not often detected. If detected, the microbes include gastrointestinal pathogens, such as *Salmonellae and Yersiniae*,(Saari et al., 1980, Mattila et al., 1982a, Careless et al., 1997) and urogenital pathogens, such as *Chlamydia trachomatis,* (Mattila et al., 1982b) although keratoconjunctivitis is a more regular feature caused by the latter (Dawson et al., 1996). All these bacteria serve as triggers of ReA as well. In addition, *Borrelia burgdorferi* has been associated with both AAU and ReA (Weyand and Goronzy, 1989, Mikkilä et al., 1997b). *Chlamydia pneumoniae*, a respiratory tract pathogen, has been associated with ReA (Saario and Toivanen, 1993, Braun et al, 1994, Hannu et al., 1999). In AS, *Klebsiella* species have been suggested to play a role in the exacerbation and/or in the development of the disease (Ebringer, 1978, Shodjai-Moradi et al., 1992, Blankenberg-Sprenkels et al., 1998) as well as in the development of AAU (White et al., 1984, Ebringer, 1988, Sprenkels et al., 1996a, Blankenberg-Sprenkels et al., 1998).

3.1.4 Differential diagnosis

The differential diagnosis of idiopathic anterior uveitis includes 1) infections caused by herpes simplex virus and varizella-zoster virus 2) infectious diseases such as syphilis and leprosy 3) uveitis entities such as Fuchs' heterochromic iridocyclitis and Posner-Schlossman syndrome 4) masquerade syndromes such as an iris melanoma or pigmentary dispersion syndrome; and 5) iritis in association with systemic diseases such as AS and related diseases, sarcoidosis, and interstitial nephritis (Rosenbaum, 1995).

Patchy or sectorial iris atrophy in connection with posterior synechiae is associated with herpes zoster ophthalmicus. A known history of recurrent keratitis helps to distinguish herpes simplex iridocyclitis from the other AU entities. Conjunctival, iris and/or angle nodule granulomas are suggestive for sarcoidosis. In addition, fatty keratic precipitates can be a sign of sarcoid uveitis, which is often bilateral and affects usually also the posterior part of the uvea. Systemic symptoms are common in sarcoidosis. Syphilis can be exluded by *Treponema pallidum* hemagglutination test (Nussenblatt et al., 1996).

Fuchs' heterochromic iridocyclitis is characterized by chronic low-grade inflammation with iris surface changes and heterochromia, fine keratic precipitates scattered over the endothelial surface of the cornea, posterior subcapsular cataract and absence of posterior synechiae. In contrast to idiopathic AAU, Fuchs patients rarely complain about the pain. The patient will be often seen for the first time by an ophthalmologist when observing floaters or when a slow but progressive lens opacification causes impairment in visual acuity (Liesegang , 1982, Nussenblatt et al., 1996).

Posner-Schlossman syndrome is by definition a glaucomatocyclitic crisis combining high IOP with iridocyclitis. This rare syndrome is recurrent and occurs in one eye episodically. Discrete, nonpigmented keratic precipitates are usually observed in the lower third of the cornea. The affected pupil is slightly dilated and inflammation in the anterior chamber may be mild to severe. The angle is open during the attacks, which tend to last from a few hours to several days. The patient usually complains of mildly blurred vision, colored halos around the lights and slight discomfort despite the high IOP. During the intervals between the attacks the IOP tends to be lower in the affected eye than in the non-affected eye. Cataract formation is not observed and there are no lesions in the vitreous or retina (Schlossman 1990).

3.1.5 Treatment and prognosis

Frequent use of topical ocular corticosteroid preparations and dilating drops are the mainstay for AAU therapy. Tapering of the topical corticosteroid is initiated as inflammation subsides. The prognosis is in the majority of cases good and there are no signs of previous inflammation between the attacks if complications have been avoided. Occasionally, periocular injection of corticosteroids is needed to control severe AAU. In most severe cases brief courses of oral corticosteroids are the drug of choice (Rosenbaum, 1995).

Oral nonsteroidal anti-inflammatory drugs such as indomethacin and ibuprofen have been used by some to avoid recurrences or severe forms of AAU. The effects of such drugs on the eye disease have not been studied in detail. All in all, one must consider the long-term side effects and expense of such drugs with the possible benefit (Rosenbaum, 1995).

Sulfasalazine has been used to taper the inflammation in wide range of SpAs. For patients with HLA-B27-associated AAU sulfasalazine may decrease the recurrence rate and intensity of the eye inflammation (Breitbart et al., 1993, Dougados et al., 1991a).

If the patient is suffering from ReA prolonged antibiotic treatment against the causative microbe has been shown to shorter the duration of Chlamydia arthritis (Lauhio et al., 1991) or prevent the development of ReA (Bardin et al., 1992, Hannu et al., 2002). Such a therapy has not been shown to be effective in AAU (Wakefield et al., 1999).

3.2 SPONDYLOARTHROPATHIES

3.2.1 Diagnostic criteria

Spondyloarthropathies (SpAs) are a heterogeneous group of diseases characterized by an association with the cell surface antigen HLA-B27, sacroilitis and spondylitis, inflammatory peripheral arthritis, insertional tendinitis (enthesopathy), and the absence of rheumatoid factor and nuclear antibodies (Moll et al., 1974, Wright and Moll, 1976). Individual conditions that overlap to form SpAs include ankylosing spondylitis (AS), Reiter's syndrome/reactive arthritis (ReA), enteropathic spondylitis (Crohn's disease and ulcerative colitis), psoriatic arthropathy, juvenile ankylosing spondylitis, and seronegative enthesopathic arthropathy syndrome. These conditions frequently co-exist with uveitis (Calin 1998). However, there is a wide spectrum of symptoms and findings suggesting for afore mentioned diseases which do not fullfill the classical criteria. Taken this into account, the European Spondyloartropathy Study Group has made the following classification criteria which also include undifferentiated forms of SpA: inflammatory spinal pain or synovitis (asymmetric or predominantly in the lower limbs), together with at least one of the following: positive family history, psoriasis, IBD, urethritis, acute diarrhea, alternating buttock pain, enthesopathy, or sacroiliitis as determined from radiography of the pelvic region (Dougados et al., 1991b).

3.2.2 Clinical features

A typical symptom of AS is persistent low back pain that lasts more than three months. Back stiffness in the morning, which improves with exercise and back pain, which wakes the patient up at nighttime and radiates to the hip and buttocks are universal. The radiological findings in sacroiliac joints may show mild changes such as sclerosis of the periarticular bone with narrowing and irregularity of the joint space or widespread progressive changes such as ankylosis and eventually the formation of bamboo spine in the lumbar area observed in the lumbosacral radiographs. Joints in lower limb and tendon insertions can be variably involved and asymmetrically painful, stiff or swollen. Indeed, plantar fasciitis, inflammation of intercostal muscle insertions, or achilles tendinitis may be the manifest signs of the disease (van der Linden et al., 1984a). Aortic root inflammation and cardiac conduction defects in association with AS occur rarely (Qaiyumi et al., 1985). Patients with peripheral arthritis are at increased risk of developing AAU (Maksymowych et al., 1995). At least 25% of patients with AS will develop AAU (Wakefield et al., 1991).

The term ReA was first introduced by Ahvonen and co-workers in 1969 to describe an inflammatory arthritis distant in time and place from the original mucosal infection (Ahvonen et al., 1969). Soon after that the association between HLA-B27 antigen and ReA was discovered (Aho et al., 1973, Aho et al, 1974). In recent years microbial antigens, including nucleic acids of the triggering microbes, have been detected in the joints of patients with ReA. The current definition of ReA has been modified as asymmetrical inflammatory oligo- or monoarthritis predominantly affecting the lower limbs in connection with the evidence of preceding infection (Kingsley and Sieper, 1996). The joint inflammation develops typically within one to two weeks after the infectious insult (Thompson et al., 1995). Most patients recover within three to five months (Hannu and Leirisalo-Repo, 1988), but 15-30% of the patients with ReA will have chronic arthritis and/or sacroiliitis. The chronic course of the disease tends to be associated with the HLA-B27 positivity (Leirisalo-Repo and Suoranta, 1988). ReA triggered by enteric infections tends to affect both men and women equally in contrast to genitourinary forms where there is a male predominance (Leirisalo et al., 1982, Samuel et al., 1995; Calin, 1998). The clinical picture of ReA is much the same independent of the causative agent. One to several joints may be involved, and the lower extremities are often involved. Inflammatory low back pain and sacroiliitis are common features (Hannu and Leirisalo-Repo, 1988). In addition, enthesopathies, conjunctivitis, keratitis, AAU, urogenital tract or mucocutaneous lesions may be observed (Rosenbaum, 1995).

About 10% of patients with psoriatic skin changes have also articular manifestations. In the most typical form of the disease the distal interphalangeal joints are affected and nail changes are evident. Other forms include sacroiliitis or spondylitis, pauciarticular peripheral disease, even symmetric peripheral disease resembling rheumatoid arthritis or in rare cases arthritis mutilans affecting few digits. Approximately 20 to 40% of patients with psoriatic arthritis are HLA-B27 positive. (Rosenbaum, 1995). Further, 7% of patients with psoriatic arthritis are reported to develop AAU (Lambert and Wright, 1976; Vinje et al., 1983).

Arthritis in association with IBD including ulcerative colitis and Crohn’s disease may present as sacroiliitis, peripheral arthritis in connection with various mucocutaneus symptoms and/or uveitis in addition to the gastrointestinal symptoms such as abdominal pain, diarrhea and presence of blood on stool (Mielants and Veys, 1998). Although diagnosis is ascertained by gut biopsy, the histological changes in the gut may be indistinguishable between IBD and other SpAs (Mielants et al., 1987; Simenon et al., 1990). Uveitis occurs in approximately 2% of patients with IBD and up to 11% of patients with IBD and sacroiliitis (Wright et al., 1965; Billson et al., 1967; Greenstein et al., 1976; Knox et al., 1984). The relationship between HLA-B27, AAU and IBD has not been thoroughly studied. In one retrospective study 46% of uveitis patients with IBD were HLA-B27 positive (Lyons

and Rosenbaum, 1997). Interestingly, uveitis in association with ulcerative colitis tends to have similar characteristics as idiopathic AAU in contrast to uveitis in association with Crohn's disease the latter been frequently bilateral, posterior, insidious in onset, and/or chronic (Lyons and Rosenbaum, 1997).

3.3 HLA-B27 AND DISEASE SUSCEPTIBILITY

HLA-B27 is distributed throughout Eurasia, but it is virtually absent among the genetically unmixed native populations of South America, Australia, and among equatorial and southern African Bantus and Sans (Bushmen). In striking contrast, it has a very high prevalence among the native peoples of the circumpolar arctic regions of Eurasia and North America. In Finland the prevalence is about 14%, among the highest in Europe (Khan, 1995). The association between HLA-B27 and AS is the second strongest relation known among HLA and disease susceptibility. Classically the relative risk of HLA-B27 for AS is mentioned as 69, for the ReA as about 25 and for AAU as 8 (Tiwari and Terasaki, 1985). So far at least 23 subtypes of HLA-B27 have been identified differing from each other mainly by the peptide binding site (Ball and Khan, 2001, Garcia-Fernandez et al., 2001). Most subtypes, although of varying degrees, are associated with the increased risk for SpAs (López de Castro, 1998). Interestingly, in China the subtype B*2704 is frequent and the prevalence of SpA is high. In contrast, native Indonesians mostly have subtype B*2706 and Sardinians B*2709 and SpA is rarely seen in these populations (Feltkamp et al., 2001). Clinical studies have shown that 35% to 70% (with an average of 50%) of patients with AAU have HLA-B27 antigen. Of this group, more than 50% will have some form of SpA including AS, ReA, arthritis in association with inflammatory bowel disease, and undifferentiated SpA (Rosenbaum 1992). On the other hand, over 90% of the AS patients posses HLA-B27 antigen in clinical materials (Brewerton et al., 1973a). Population studies, however, show that only 43% of the AS cases are HLA-B27 positive (van der Linden et al., 1984b). The prevalence of AS in general population is estimated to be 0.1 to 0.3% and 1-3% in the HLA-B27-positive population (Cohen et al., 1985, Linssen et al., 1991, Kaipiainen-Seppänen et al., 1997).

HLA-B27 is a major histocompatibility complex (MHC) class I molecule expressed on nearly all nucleated cells and participating in endogenous antigen presentation to specific T lymphocytes. In contrast, class II molecules are expressed on extracellular antigen processing and presenting cells such as macrophages, B lymphocytes and dendritic cells (Forrester et al., 1999). However, Pfeifer et al (1993) have reported that phagocytosed bacterial derived antigens have been presented also by MCH class I molecule. Intracellular proteins are degraded in the proteasome and bound to the MHC class I molecule, including HLA-B27, in the endoplasmic reticulum. The peptides are bound in grooves formed by the α_1 and α_2 domains of the MHC class I molecule, and anchored at specific sites to the β pleated sheets that form the floor of the groove. MHC-peptide complexes are then transported to the

plasma membrane where they are oriented in such a way that the peptide is exposed to the extracellular compartment for interaction with $CD8^+$ T cells.

Most endogenous peptides which bind to HLA-B27 are 9 to 10 amino acids long containing arginine at position 2 (Jardetzky et al., 1991). Also peptides with other residues have been isolated from HLA-B27 (Simmons et al., 1997). The peptide is usually arched in the middle of the groove with its amino acid residues projecting outwards to the T cell receptor (TCR). These exposed residues determine the specificity of the reaction with the TCR. All T cells have receptors for peptide-MHC complexes, but a subset of T cells populating mucosal epithelium has been shown to possess TCRs, which appear to recognize heat shock proteins (proteins expressed in ”stressed” cells and highly conserved across species). In addition, both cell-specific accessory molecules and nonspecific adhesion molecules are involved in the activation of T cells (Forrester et al., 1999).

Microimmunofluorescence test (MIF) and polymerase chain reaction (PCR) give rather equal results in terms of specificity. However, the down-regulation of the expression of HLA-B27 has been shown in patients with ReA which could result in false negative typing if only cell surface expression is studied (Kirveskari et al, 1997). Also, transient loss or masking of HLA-B27 epitopes, has been suspected on patients with AS (Amor et al., 1978; Neumuller et al., 1993). In vivo evidence of the decreased expression of HLA-B27 during bacterial infections has been lacking so far, but transient decrease of HLA-B27 epitopes during chronic *Klebsiella* infection has been observed (Kirveskari et al., 1999). In our study nine patients with idiopathic AAU originally tested by microlymphocytotoxcity test to be HLA-B27 negative did not have HLA-B27 gene ascertained by PCR.

In considering the pathogenesis of SpA, the role of the most important predisposing gene, HLA-B27, may be more complex than earlier thought. Initial hypotheses were based on the assumption of HLA-B27 mediating arthritis/uveitis through its physiologic function as an antigen-presenting molecule. Recently, a growing body of evidence has cumulated connecting HLA-B27 also with a role unrelated to antigen presentation. The theories proposed to explain the mechanism by which HLA-B27 influences disease susceptibility are presented in table 1.

Table 1. Hypotheses for the role of HLA-B27 in disease susceptibility

Theory	Reference
Antigen presentation	
1) Molecular mimicry	Schwimmbeck et al., 1987
2) Arthritogenic peptide	Benjamin and Parham, 1990
3) Promiscuous peptide	Davenport, 1995
4) Reactive thiol hypothesis (modified self)	Whelan and Archer, 1993
5) Heavy chains of HLA-B27	Khare et al., 1996
Other functions	
1) Enhanced innate immune responsiveness	Repo et al., 1990
2) Favouring the maintenance of arthritogenic microbes	Kapasi and Inman, 1992; Virtala et al., 1997
3) Altered response to invasion of arthritogenic microbes	Ikawa et al., 1998
4) Misfolding	Colbert et al., 2000

The molecular mimicry theory suggests antigenic similarities and cross-reaction between bacterial derived peptides and HLA-B27 molecule derived self-peptides. This would result in the production of autoantibodies and/or cytotoxic T cell reaction (Schwimmbeck et al., 1987). Interestingly, the difference between the SpA-associated and non-SpA-associated HLA-B27 subtypes is limited to only two amino acid residues (114 and 116) at the bottom of the peptide-binding groove of HLA-B27 (Feltkamp et al., 2001). A plasmid encoded in *Shigella* antigen has been suggested to mimic HLA-B27 derived self-peptides (Stieglitz et al., 1989). Further, an HLA-B27-derived peptide mimicking particularly a region of the DNA primase from *C. trachomatis* has been demonstrated recently (Ramos et al., 2002). In accordance with this HLA-B27 positive cells infected with ReA-inducing bacteria have been shown to express increased amounts of certain self-peptides (Ringrose et al., 2001).

The arthritogenic peptide hypothesis serving as well for uveitogenic peptide model is based on the presumption that a bacterial peptide is antigenically cross-reactive with a self-peptide expressed in joints or anterior uveal tract. The bacterial peptide is presented to CD8 cells by HLA-B27. After infection, sensitized CD8 cells could recognize self-peptides expressed in joints or anterior uveal tract, and cause an autoimmune response damaging host tissues (Benjamin and Parham, 1990).

The promiscuous peptide theory relies on the finding that HLA-B27 molecule possesses a short sequence similar to arthritogenic/uveitogenic bacterial peptides. HLA-B27 derived ”promiscuous” peptides were suggested to be presented by class II HLA molecules to CD4 cells inducing autoimmunity (Davenport, 1995). However, later findings obtained from transgenic mice, have refuted this theory (Khare et al., 1998a). Indeed, HLA-B27 molecule has the capacity to bind self-peptides and present them to CD8 cells (Scofield et al., 1995). The presentation of HLA-B27 derived self-peptides is not likely to play a important role in the pathogenesis of SpA, since they are naturally presented by several HLA-B27 subtypes, also those that are not associated with the disease (García et al., 1997).

The reactive thiol hypothesis is based on the fact that the peptide-binding groove of the HLA-B27 molecule contains an unpaired cysteine at position 67 with a potentially reactive thiol group. This has led to the idea that the oxidation and subsequent alteration of the peptide-binding groove may modify the peptide binding and presentation by HLA-B27, or altered antigenicity of HLA-B27 itself (Whelan and Archer, 1993). However, it remains a mystery why only some of the HLA-B27 positive individuals develop the disease, although they all possess the reactive thiol group.

High innate immune responsiveness is suggested to be associated with HLA-B27 antigen. In an acute inflammatory reaction, neutrophils are considered to cause tissue injury by both liberating lysosomal enzymes and generating toxic oxygen-derived free radicals. Studies of patients with ReA triggered by yersinia enteritis (Leirisalo et al., 1980) and of patients with AS (Pease et al., 1982) have revealed that HLA-B27 positive neutrophils obatained from either patients or healthy subjects show higher chemotaxis *in vitro* than do neutrophils obtained from healthy subjects who are HLA-B27 negative. The hyperreactive neutrophils could trigger an inflammation cascade and render the subjects susceptible to exaggerated tissue injury (Repo et al., 1984). Later on, it has been shown that neutrophils from HLA-B27 negative patients with AS show enhanced chemotactic responsiveness (Pease et al., 1984). Moreover, neutrophils from HLA-B27 negative patients with previous yersinia arthritis tended to be more reactive than neutrophils from HLA-B27 negative controls (Repo et al., 1988). These findings give credence to the view that enhanced responsiveness is rather associated with the disease than HLA-B27 antigen itself (Repo et al., 1990).

One interesting theory proposes that the heavy chains of HLA-B27 mimics class II HLA molecules. In studies with HLA-B27 transgenic mice it has been shown that in the absence of mouse β_2-microglobulin these mice develop spontaneous inflammatory arthritis when removed from a germ-free environment (Khare et al., 1995). Further, it has been suggested that the HLA-B27 molecule together with human β_2-microglobulin forms unstable peptide-MHC complexes dissociating on the cell surface

and leading to the expression of free and empty heavy chains on the cell surface and presenting an exogenous antigen to CD4 cells (Khare et al., 1996, Khare et al., 1998a, Khare et al., 1998b).

Kapasi and Inman (1992) were the first to report that HLA-B27 may affect directly on the interaction between host cells and microbes. The invasion of gram-negative bacteria was shown to be decreased in the HLA-B27-transfected murine fibroblast L cell line. Moreover, the invasion was enhanced when HLA-B27 expression was diminished (Kapasi and Inman, 1994). However, the level of invasion of SpA-triggering bacteria into HLA-B27-positive and -negative cells might not be the main issue in the pathogenesis of SpA. In several studies, HLA-B27 seems to interfere with intracellular elimination of SpA-triggering bacteria both in transfected cell lines. The elimination of *S. enteritidis* in monocytic and fibroblast cell lines has been shown to be decreased possibly influenced by impaired nitric oxide production in the latter case (Laitio et al., 1997; Virtala et al., 1997). Controversial results have also been reported (Huppertz and Heesemann, 1996; Ortiz-Alvarez et al.,1998) reflecting probably the variety of microbial strains and virulence in addition to differentiation of cell types used in these studies.

Evidence has cumulated that phagocytosed microbes could lead to activation of genes, which could modify the host response to infectious agents. Down-regulation of the expression of some MHC class I molecules including HLA-B27 has been reported in patients with acute Salmonella or Yersinia infection (Kirveskari et al., 1999). Furthermore, it has been shown that the invasion of Salmonella into epithelial cells induces the expression of the c-*fos* gene leading to the production of monocyte chemoattractant protein-1 in the presence of HLA-B27 (Ikawa et al., 1998).

In relation to afore mentioned findings it has been shown that HLA-B27 heavy chain tends to misfold during assembly (Mear et al., 1999). Protein misfolding can influence intracellular signaling pathways (Mear et al., 1999; Colbert, 2000a; Colbert, 2000b) and could be responsible for the non-antigen presentation effects. HLA-B27 misfolding and accumulation might contribute an endoplasmic reticulum stress response leading to nuclear factor κB (NF-κB) activation. This could stimulate synthesis of proinflammatory cytokines such as TNF-α in monocytes and macrophages. Interestingly, monocytes cell lines expressing HLA-B27 have enhanced NF-κB activation and TNF-α production compared with control monocytes upon *Salmonella* LPS stimulation (Penttinen et al., 2000).

3.4 GRAM-NEGATIVE BACTERIA

3.4.1 Structure and functions of the outer membrane

Bacteria are classified as Gram-positive or Gram-negative depending on an outer membrane and its staining properties. Contrary to Gram-positive Gram-negative bacteria possess an outer membrane. It consists mainly of lipopolysaccharide (LPS), but also phospholipids and proteins. Further, outer membrane protects bacteria from host defense. On the other hand, many structures of the outer membrane induce a variety of symptoms in the host and modulate immune responses (Koebnik et al., 2000).

LPS of the outer membrane is an important antigenic structure and a part of the defense mechanism of the cell wall. In addition, it has a marked toxic influence on the host and for this reason it is called endotoxin. LPS consists of three components: lipid A, core oligosaccharide, and O-antigen (Morrison and Ulevitch, 1978). Lipid A is practically the only lipid component in the outer surface of outer membrane. O-antigen is located at the outermost part of the LPS and in addition in the outer surface of the cell, and is indeed one of the most important surface antigenic structures of bacteria. Moreover, it protects the bacteria from phagocytosis. Many of these bacteria have a sheltering capsule. Others like *Chlamydiae* species are intracellular pathogens protected from the serum antibodies, complement cascade, and phagocytosis. LPS is an important cause of morbidity during infections with gram-negative bacteria. It is the major cause of shock, fever, and other pathophysiologic responses to bacterial sepsis (Nathanson, 1989). The manifold effects of LPS include activation of the monocytes and polymorphonuclear leukocytes, leading to the up-regulation of genes of various cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-α, as well as degranulation, activation of complement via the alternative pathway, and direct influence on vascular endothelium. The cellular effects of LPS are the result of interactions with specific cell receptors such as CD 18-CR3, a specific LPS scavenger receptor on macrophages and lymphocytes. A circulating LPS binding protein has been identified. Binding by the LPS-binding protein complex with the CD14 molecule on the macrophage surface results in activation. CD14 molecule serves as a cell surface component of a receptor complex through which the macrophage recognizes the presence of microbial components such as LPS (Ziegler-Heitbrock and Ulevitch, 1993, Henneke and Golenbock, 2002).

3.4.2 *Chlamydia pneumoniae* and *C. trachomatis*

Both *C. pneumoniae* as well as *C. trachomatis* infections are common in general population. It has been estimated that almost everybody go through upper respiratory tract infection caused by *C. pneumoniae* two to three times in their lifetime commonly starting at the age of 5 to 14 years (Kuo et al., 1995). In young adults the pneumonia is associated with the primary infection causing mild symptoms but in older age the pneumonia is likely to be a reinfection causing even life threatening symptoms and leading to complications such as erythema nodosum, meningitis, hepatitis (Sundelöf et al., 1993), carditis (Gran et al., 1993), lymphadenitis (Machi and Okino, 1997) and ReA (Gran et al., 1993, Hannu et al., 1999). *C. pneumoniae* infection has been associated even with the pathogenesis of atherosclerosis, myocardial infarcts and destruction of cardiac valves during inflammation (Leinonen and Saikku, 2002) as well as predisposing to the development of asthma (Johnston, 2001) and chronic obstructive pulmonary disease (Hayashi, 2002). Like *C. pneumoniae*, *C. trachomatis* is an intracellular pathogen. Serotypes A, B, Ba, or C are associated with the classic blinding endemic trachoma of developing countries, which is spread "eye to eye" (Dawson et al., 1996). Serovars D through K are capable of inducing persisting infection in connection with atypical or minor genitourinary or abdominal symptoms. Approximately 3% of the women in fertile age and 1-2% of men are symptom free carriers of *C. trachomatis*. Chronic infection has been shown to produce complications such as salpingo-ophoritis, ectopic pregnancy and infertility (Mardh and Novikova, 2001). These sexually transmitted strains of *C. trachomatis* can produce an eye disease resembling the early inflammatory phases of endemic trachoma but usually without the severe conjunctival scarring (Dawson et al., 1996). The immunopathogenetic mechanism of chlamydial infections has not been resolved yet. However, it has become evident that antibodies are not likely to have a major role in the clearance of chlamydial infection although they may protect the host from the reinfection caused by the same immunotype (Beatty et al., 1993; Schachter, 1999). A key issue in chlamydial diseases is whether the pathologic mechanisms are associated with an enhanced immune response mediating tissue destruction through cytotoxic reactions (Ward, 1999), or whether they are related to the Th2 type of response that eventually leads to the partial or temporary suppression of an effective antichlamydial response (Th1 response) (Yang et al., 1996; Yang et al., 1999). In both models, chlamydial heat shock protein 60 (Hsp60) has been shown to be the key antigen.

3.4.3 Heat shock proteins

Hsps are highly conserved proteins present among both prokaryotes and eukaryotes. There are four main groups of structurally related Hsps based on their molecular weights and the individual members of each family share 40-95% amino acid homology between different species (Buchner et al., 1998;

Lindquist and Craig, 1988; Cerrone et al., 1991). The ability of Hsps to (1) chaperone peptides, including antigenic peptides; (2) interact with antigen-presenting cells through a receptor; (3) stimulate antigen-presenting cells to secrete inflammatory cytokines; and (4) mediate maturation of dendritic cells makes Hsps a unique starting point for generation of immune responses (Basu et al., 2000). In addition to chlamydial infections, a number of infectious diseases are associated with activated humoral and cellular responses to microbial Hsps (Kaufmann and Schoel, 1994; Zugel and Kaufmann, 1999). Owing to the high amino acid and structural homology of the Hsps between different species, the immune memory, either humoral- or cell-mediated, is considered not to be limited only to the microbe in question but also involve other, possible more virulent pathogens that subsequently invade the host (Kaufmann and Schoel, 1994). On the other hand, the immune response once initiated by the microbial Hsp may also be evoked against autologous Hsp epitopes. Recognition of the self-Hsp may subsequently break down the immune tolerance against these cross-reactive structures and convert the protective immune responses into pathological ones (Kaufmann and Schoel, 1994). In this respect, the chlamydial Hsp60 has been a target of research interest during the past decade (Ward, 1999; Neuer et al., 2000).

3.4.4 Antigen persistence

During the past two decades an increasing body of evidence has accumulated to support the theory that microbes triggering ReA are persisting and/or consistently distributed from gut or mucosal sites in the host. Prolonged antibody responses to Salmonella (Mäki-Ikola et al., 1991; Mäki-Ikola and Granfors, 1992) and Yersinia species (Granfors et al., 1980; Granfors et al., 1989b) have been observed in ReA. Furthermore, prolonged (Calcuneri et al., 1981) and elevated antibody levels against Klebsiella (Mäki-Ikola et al., 1998; Ebringer, 1992; Nissilä et al., 1994; Mäki-Ikola et al., 1995) in AS and especially in patients with the axial form of the disease (Mäki-Ikola, et al., 1997a) or in association with AAU (Mäki-Ikola et al., 1995) have been observed. These findings have been presented as evidence of the role of Klebsiella in AS. Further, gram-negative bacterial antigens, in addition to DNA and RNA, have been found within synovial membrane (Schumacher et al., 1988; Merilahti-Palo et al., 1991; Hammer et al., 1992; Taylor-Robinson et al., 1992), synovial fluid cells (Keat et al., 1987; Granfors et al., 1989a; Granfors et al., 1990; Viitanen et al., 1991; Granfors et al., 1992; Bas et al., 1995; Nikkari et al., 1999), and peripheral blood cells (Granfors et al., 1990; Granfors et al., 1998; Schumacher et al., 1997; Schumacher et al., 1999) in patients who had been infected with that agent and developed ReA. However, contradictory findings of intra-articular chlamydial (Poole et al., 1992), Yersinia and Salmonella DNA (Gaston et al., 1999, Wilkinson et al., 1999, Nikkari et al., 1999) have been reported. Moreover, chlamydial DNA have even been detected in the joints of patients with RA as well as asymptomatic subjects (Schumacher et al., 1999).

3.5 IMMUNE DEFENCE MECHANISMS

3.5.1 Immune privilege of the eye

The eye participates in immune responses, but under certain circumstances the expected response does not occur; this is called “immune privilege” (Forrester et al., 1999). Animal studies have shown that foreign tissues placed in the anterior chamber of eyes of immunologically intact animals may survive for long periods of time whereas similar tissues implanted subcutaneously would be instantly rejected (Kaplan and Stevens, 1974). Other privilege sites in the body include the brain, certain endocrine organs, the liver and the maternal-fetal interface (Barker and Billingham, 1997). Although all of these tissues have access to lymphatic vessels and drainage pathways to lymph nodes, the lymphatic connections to the eye are unusual. The ocular surface covered by the conjunctiva is part of the mucosal system (Brandtzaeg, 1989). In contrast intraocular space including anterior chamber is neither an integral part of the mucosal nor the skin-associated lymphoid immune system (Streilein, 1990). The intraocular space forms a unique immunosuppressive milieu comprised of cells and molecules, which interact with the rest of the body in highly distinctive ways. There are two important features making the intraocular space as an immunologically privileged site. First, blood-aqueous barrier comprised of specialized endothelial cells lining intraocular vessels regulates tightly the passage of cells and molecules from the systemic circulation into the eye. Second, the escape of cells and molecules from the eye into the rest of the body is well controlled. Most of the aqueous humor is drained through the trabecular meshwork directly into the venous circulation. Intraocular cells and molecules are rarely allowed to enter into the lymphatic system draining the internal eye. If the uveoscleral pathway is rendered patent, the possibility for direct communication between the internal eye and draining cervical lymph nodes exists (Streilein, 1995).

3.5.2 Innate immunity and adaptive immunity

Immunity is by definition the ability of the host to protect itself against a foreign organism. To do this the host requires an immune system comprising of cells and molecules to remove and destroy foreign organisms while ‘self’ molecules and cells are not attacked. Two immune systems are available to the host, innate (natural or native) immune system and the adaptive (acquired) immune system. The innate immune system is comprised of (1) physiochemical barriers such as the skin, eyelids and tears, (2) molecules normally present in body fluids such as blood, tears and aqueous humor (e.g. complement, lysozyme, antiproteases), (3) phagocytic and cytotoxic cells such as polymorphonuclear leukocytes, macrophages, eosinophilic granulocytes, natural killer cells, and (4) molecules released by cells

responding to attack and acting on other cells (cytokines), such as macrophage TNF-α. The adaptive immune system consists of (1) specific immune systems associated with barrier surfaces such as the skin immune system and the mucosa-associated immune system (2) lymphocytes with receptors that specifically recognize foreign antigens, (3) antibodies derived form B lymphocytes that specifically counteract foreign antigens, (4) lymphocyte-secreted cytokines (Forrester et al., 1999).

The innate immune system forms an alike nonspecific response to all foreign organisms and even to injury. This may be inadequate to protect the host from subsequent attacks and may lead to persistence of foreign material. Adaptive immune response is based on an immunologic memory. Each subsequent attack by the same foreign organism arouses specific and stronger immune response. The innate immunity, not dependent on prior exposure to the foreign antigen, provides an early warning, rapid-response system against most extracellular organisms. In contrast, if the pathogen resides within the host cell, as in the case of *Chlamydia* species or viruses, and incorporates to some extent into the DNA of the host cell, it may lead to the expression of the foreign antigen on the surface of the host cell in addition to the self-molecules also called self-antigens. Removal of infected cells requires a mechanism in which recognition of foreign antigens occurs in conjunction with self-antigens. This has led to the development of the adaptive immune system with a considerable degree of sophistication and variety of T and B lymphocytes . T lymphocytes are specialized in dealing with surface-bound antigens whereas B lymphocytes are specialized in dealing with soluble (extracellular) antigens. The adaptive immune system has thus been harnessed to assist the innate immune system in dealing more efficiently with extracellular organisms via B cells (Forrester et al., 1999).

3.6 PATHOGENETIC MECHANISMS PROPOSED TO PLAY A ROLE IN AAU

The basic mechanisms responsible for AAU are still unknown. Both immune complex (HLA-B27 associated) and cell mediated autoimmune processes have been proposed to explain the pathogenesis of the disease. Because of the nature of the AAU and ethical reasons, human tissue is rarely available for the research. In the course of twenty years two different types of animal models have greatly increased knowledge of the pathogenic mechanisms of anterior uveitis. In 1980, Rosenbaum et al. reported that a systemic immunization with endotoxin triggered bilateral AAU in the rat. Since then many studies concerning different events in the cascade of endotoxin induced uveitis (EIU) in rats and mice have been carried out. In the beginning of 1990s, Broekhuyse et al. reported that an acute recurrent uveitis termed “experimental melanin-induced uveitis” (EMIU) is observed when Lewis rats are immunized with bovine choroidal melanin.

3.6.1. Cellular mechanisms of AAU

Neutrophils, normally absent from the anterior uvea (McMenamin,1997), have been shown to predominate in the inflammatory site in EIU evidenced by histopathological and immunohistochemical studies (Bhattacherje et al., 1983; Cousins et al., 1984; McMenamin and Crewe, 1995). There are two peaks of the neutrophil influx into anterior uveal structures during EIU; at about 5 hours and 24 hours following endotoxin injection. Further, neutrophils can be detected in the inflammation site even 6 weeks after systemic injection. In EMIU neutrophils have been observed as well mainly in the early stages of the disease (Broekhuyse et al., 1993; Chan et al., 1994; Bora et al., 1995). Neutrophils play a key role in acute inflammation invading from the vascular system to the inflammation site and having the capability of phagocytosing non-desirable particles. They may also induce immunomodulatory effects by secreting cytokines, eicosanoids, platelet-activating factor and cationic proteins (Forester et al., 1995).

CD4-positive T lymphocytes have been shown to possess a controlling role in EMIU whereas CD8-positive T cells and B cells are present only in small numbers locally. Indeed, EMIU may be eliminated by systemic administration of anti-CD4 monoclonal antibody, but is not influenced by anti-CD8 monoclonal antibody (Smith et al., 1998a). Interestingly, active participation of T cells in EIU has also been suggested on the basis of the treatment trials. Systemic pre-treatment with monoclonal antibodies to CD4-positive T lymphocytes decreases clinical and histological inflammations findings in CH3/HeN mice with EIU (Kogiso et al., 1992). Also, tacrolimus, an immunosuppressant of which major clinical effect is directed against IL-2 induced T cell activation and proliferation, reduces both aqueous cells and histological inflammation in Lewis rats with EIU (Hikita et al., 1995).

Monocytes circulating in blood are important elements of the innate immune system. Stimulated by LPS they secrete pro-inflammatory cytokines, which induce the production of acute phase proteins, which may lead to anterior uveal inflammation and activation of adaptive immune responses (McMenamin and Crewe, 1995; Ulevitch and Tobias, 1999). As a sign of activation, monocytes' surface expression of the β_2-integrin CD11b/CD18 is increased in the acute phase of the inflammation (Prieto et al., 1994; Takala et al., 1999a). Further, monocytes weakly positive for CD14 and co-expressing Fcγ-III receptor (Fcγ-IIIR) are known to be able to produce significantly more TNF-α than other monocyte subsets (Frankenberger et al., 1996). Macrophages, which maturate from monocytes, participate in cell-mediated immunity and other inflammatory responses, tissue repair and angiogenesis, as well as in the destruction of microbes and tumor cells. An intense network of tissue macrophages covers the base of the ciliary body from iris base to pupil margin, and extends along the vessels of ciliary processes in the rat. In the ciliary body intraluminal monocytes are strongly adhered

to vascular endothelium. Same kind of network of macrophages is likely to exist in human anterior uvea (McMenamin et al., 1994). In EIU monocytes start to migrate in iris vessels 2 hours after injection of LPS and by 24 hours they are displayed widely among the tissue macrophages in anterior uvea (McMenamin and Crewe, 1995). In EMIU infiltrating macrophages are detected also from the beginning of the inflammation and are suggested to act similarly in this disease model (Kim et al., 1995).

Dendritic cells capable to activate naive T cells and express MCH class II molecules have been identified in the anterior uvea (McMenamin and Crewe, 1995). They have been detected everywhere in the iris and ciliary body stroma particularly at the border of the anterior chamber. Moreover, some dendritic cells are situated in close connection with ciliary epithelial cell junctions which contribute blood-aqueous barrier; an ideal site for hunting intraocular antigens. Similar mechanisms are likely to exist in human tissue as well (McMenamin et al., 1994). Interestingly, from 2 hours onwards after the LPS injection in EIU dendritic cells begin to convert into pleiomorphic or round variety. This is followed by increase of the cell amount and turnover rate (McMenamin and Crewe, 1995). Intensification of immune surveillance due to an increased antigen sampling and processing is an adaptive response to inflammation. However, this may lead to exposing intraocular antigens to systemic immune system and result in autoimmune disease as discussed by Smith and co-workers (Smith et al., 1998d).

3.6.2 Molecular mediators of AAU

3.6.2.1 Adhesion molecules

Vascular endothelial surface glycoproteins named adhesion molecules control the movements of leucocytes through vascular endothelium into inflammatory sites in four stages: rolling; arrest; firm adhesion; and transmigration. Adhesion molecules are divided into four different structural groups, namely selectins which mediate the rolling; integrins (lymphocyte function-associated molecule LFA-1), members of the immunoglobulin gene superfamily (intercellular adhesion molecule ICAM-1) participating in leukocyte adhesion and transmigration; and sialomucins mediating both rolling and adhesion stages (Carlos and Harlan, 1994). Many of the events mentioned afore have been observed in studies concerning EIU (Whitcup et al., 1992; Whitcup et al., 1993; Carlos and Harlan, 1994; Whitcup et al., 1995; Kanagawa et al., 1996; Whitcup et al., 1997; Suzuma et al., 1997) and in EMIU (Chan et al., 1994; Kim et al., 1995). Although expression of adhesion molecules during AAU has never been examined in humans, members of the selectin, integrin and immunoglobulin gene superfamily have

been detected in iris biopsy specimens obtained from patients with chronic anterior uveitis and panuveitis. These adhesion molecules have not been found in uninflamed control eyes (Wakefield et al., 1992).

3.6.2.2 Proinflammatory cytokines

Cytokines regulate the immune response by inducing the activation, proliferation and differentiation of a variety of cells in addition to controlling the production of other cytokines. They are low-molecular-weight proteins and glycoproteins that act through specific cell surface receptors. TNF-α and interleukin-1 (IL-1) are likely to play a key role in the pathogenesis of EIU (Yoshida et al., 1994, Planck et al., 1994; De Vos et al., 1994a; De Vos et al., 1994b; De Vos et al., 1996) and the former as well in EMIU (Woon et al., 1998). A variety of cells can secrete TNF-α as a response to infectious and inflammatory agents including LPS (Akira et al., 1990). In several studies markedly elevated mRNA levels for TNF-α have been detected in rats in iris-ciliary body during EIU 3 hours and again 24 hours after injection (Yoshida et al., 1994, Planck et al., 1994; De Vos et al., 1994a; De Vos et al., 1996). Further, levels of the TNF-α in serum and aqueous show similar peaks at 4 hours and about 24 hours (De Vos et al., 1994b). The first peak is thought to be produced by tissue macrophages responding to endotoxin, while infiltrating cells may be responsible for production of the second rise in TNF-α levels. In accordance with this, the mRNA expression of TNF-α was up-regulated in contrast to other cytokines, i.e. interferon gamma (IFN-γ), interleukin-10 (IL-10), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), in the iris and ciliary body during EMIU (Woon et al., 1998). As a pro-inflammatory cytokine in uveitis, TNF-α is likely to induce adhesion molecules and MCH class II antigens expression. It may stimulate neutrophils and macrophages for synthesis of prostaglandins, nitric oxide and other cytokines like IL-6 (Akira et al., 1990).

Anterior uveitis mimicking EIU can be triggered in rodents and rabbits by intravitreal injection of IL-1 (Ferrick et al., 1991). Like TNF-α, IL-1 has a central role in the inflammatory process as an activator of leukocytes, monocytes, and endothelial cells. IL-1 may be produced by resident tissue macrophages and also by infiltrating cells as a direct response to LPS. IL-1 has especially the ability to induce adhesion molecule expression on endothelial cells and also to promote prostaglandin synthesis by these cells (Akira et al., 1990).

IL-6 has proinflammatory activity for example on lymphocytes and macrophages but recent evidence refers to participation in limiting tissue damage (Forrester et al., 1999). IL-6 may be produced by a number of cells including neutrophils, macrophages and lymphocytes, and by the influence of TNF-α and IL-1 in EIU. Moreover, IL-6 gene has been shown to be activated directly by LPS (Akira et al.,

1990). Indeed, EIU may be induced by intravitreal injection of IL-6 (Murray et al., 1990). However, this cytokine's activities can be covered by others, i.e. TNF-α, interleukin-1 beta (IL-1β), IL-10, IFN-γ, monocyte chemotactic protein 1 and macrophage inflammation protein, and it is not essential for induction of EIU (De Vos et al., 1994a).

T lymphocytes are the main producers of IFN-γ, which mainly triggers macrophage activation (Young and Hardy, 1993). In contrast to other proinflammatory cytokines, low but detectable levels of IFN-γ can be measured in normal eyes and it may induce activation of the first infiltrating monocytes in EIU. Subsequently, activated lymphocytes may secrete more proinflammatory cytokines to stimulate macrophages (Planck et al., 1994; De Vos et al., 1994a; De Vos et al., 1996).

3.6.2.3 Immunomodulatory cytokines

IL-4 produced by T cells suppresses macrophage and monocyte activities and in the same time may exacerbate and suppress various lymphocyte functions (Brown et al., 1997). Unexpectedly, IL-4 deficient mice have been shown to develop significantly milder EIU compared with normal phenotype controls (Smith et al., 1998b). Also, in some experimental studies, macrophages and monocytes pre-treated with IL-4 secrete TNF-α and IL-6 in response to endotoxin (D'Andrea et al., 1995; Kambayashi et al., 1996). This may be explained by following observations: IL-10 may inhibit IL-4 activity (Kambayashi et al., 1996), and in addition, IL-4 is known to affect expression of adhesion molecules (Masinovsky et al., 1990; van den Berg et al., 1996) and up-regulation of MHC II molecules on macrophages and monocytes (Te Velde et al., 1988).

A rise in messenger ribonuclein acid (mRNA) levels of IL-10 in aqueous humor has been measured before the onset of EIU (De Vos et al., 1994a). IL-10 is produced by lymphocytes and macrophages and it inhibits antigen-specific activation of Th1 lymphocytes thereby suppressing cytokine production of Th1 cells (De-Waal-Malehyt et al., 1992). The down-regulation effect by IL-10 is dose dependent in a manner that low doses exacerbate the inflammation and high doses inhibit it (Rosenbaum and Angell, 1995). Interleukin-12 (IL-12) has as well been observed to have both pro-inflammatory and immunosuppressive effects during EIU. This phenomenon was demonstrated with C3H/HeN mice, which were predisposed to anti-IL-12 monoclonal antibodies systemically and intravitreally. EIU was enhanced in the former and inhibited in the latter case (Whitcup et al., 1996).

Transforming growth factor-β (TGF-β) suppresses T cell proliferation, stimulates T cell inhibitory functions, and down-regulates macrophage activation. A variety of cells are able to produce the latent form of TGF-β, which is then converted to mature form (TGF-β1 and TGF-β2) by the influence of

proteolytic enzymes and acidic environment (Cohen and Cohen, 1996). TGF-β1 and TGF-β2 diminish the inflammation in the onset of EIU, but in continuing disease the latter is suggested to play a dominant role as an immunosuppressant. Lower levels of TGF-β1 and its mRNA have been detected in eye with recurrent disease in EMIU (Li Q et al., 1996). Indeed, in the C3H/HeN mouse model of EIU, TGF-β1 injected intraperitoneally has been shown to diminish the inflammation (Peng et al., 1997).

3.6.2.4 Chemokines, eicosanoids, nitric oxide, matrix metalloproteinases and fas ligand

Chemokines possess leukocyte chemoattractant activity (Proost et al., 1996). They are divided into two groups, namely CXC chemokines which attract neutrophils and CC chemokines which attract mononuclear cells secreted by phagocytes and lymphocytes. In patients with AAU a rise in the levels of both CC and CXC chemokines in aqueous humor has been shown during active inflammation (Verma et al., 1997).

Eicosanoids (prostaglandins, thromboxanes and leukotrienes) are known to have a profound influence on hormonal and inflammatory activity. Elevated levels of thromboxane B_2, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) have been measured by radio-immunoassay during EIU. Thromboxane B_2 is an inactive metabolite of thromboxane A_2, which is secreted by intravascular thrombocytes in the first place. It stimulates neutrophils to adhere to vascular endothelium. At the site of the inflammation the initial elevation of this mediator may be due to vascular leakage. However, also neutrophils are capable of producing thromboxane A_2 later. Prostaglandins disturb vascular permeability and may be able to break down the blood-aqueous barrier (Herbort et al., 1988; De Vos et al., 1994b). In accordance with this prostaglandins have been shown in the anterior chamber in human AAU (Whitelocke et al., 1973). Indeed, topical indomethacin as a prostaglandin synthesis inhibitor may reduce the inflammation in AAU although in lesser extend than corticosteroids (Sand et al., 1991). Acting as a neutrophil chemoattractant the LTB_4 may enhance accumulation of these cells in the anterior segment during EIU (Herbort et al., 1988).

Nitric oxide (NO) is an oxygen free radical released from L-arginine by nitric oxide synthase (NOS). Synthesis of NO by infiltrating macrophages and neutrophils and/or by vascular endothelium is induced by LPS or cytokines like TNF-α and IL-1 (Lowenstein et al., 1994) and increases during EIU and EMIU (Jacquemin et al., 1996; McMenamin and Crewe, 1997; Kim et al., 2001). NO is capable of breaking blood-aqueous barrier at least in experimental models (Jacquemin et al., 1996; McMenamin and Crewe, 1997). Indeed, NO and PGE_2 synthesis inhibitors have been shown to have synergistic effect on uveitis triggered in the rabbit by intravitreal injection of endotoxin (Bellot et al., 1996).

However, certain observations based on animal models are in agreement with a theory that the inflammatory activity of NO can be replaced by other molecular mediators (Smith et al., 1998b).

Matrix metalloproteinases are enzymes with influence on regeneration of connective tissue, and on the other hand tissue destruction during inflammation (Kahri and Saarialho-Kere, 1997). All three subgroups: collagenases, gelatinases and stromelysins have been identified from normal human aqueous humor (Ando et al., 1993). The imbalance between the metalloproteinase activity and its inhibitors has been suggested to be the major cause promoting tissue damage in uveitis (Di Girolamo et al., 1996). Resident uveal fibroblasts and infiltrating inflammatory cells are thought to be responsible for enzyme activity triggered by cytokines like TNF-α and IL-1 in normal and inflamed aqueous humor (Kahri and Saarialho-Kere, 1997).

Fas is a cell surface molecule expressed by neutrophils, lymphocytes, monocytes, and macrophages. The stimulation of Fas by Fas ligand (FasL) promotes programmed cell death or apoptosis of these cells (Nagata and Goldstein, 1995). Apoptosis has been shown to be an important feature of the spontaneous resolution of both the EIU and EMIU (Smith et al., 1998c). Further, infiltrating mononuclear cells have been observed to be eliminated early in the disease process while neutrophils survive. Interestingly, cross-linking of adhesion molecule CD11b, endothelial transmigration and LPS are all able to block Fas-stimulated signaling for neutrophil apoptosis (Watson et al., 1997). This phenomenon has led to an idea of neutrophils being the primary suspects for tissue destruction, even in antigen triggered disease (Smith et al., 1998d).

3.6.3 Summary of pathogenic mechanisms in experimental animal models of AAU

EIU is believed to result from the release of a variety of mediators by activated inflammatory cells. Systemically injected LPS may act directly on resident tissue macrophages causing the production of the most important pro-inflammatory cytokines TNF-α and IL-1. In addition to intrinsic pro-inflammatory effects these cytokines control the expression of IL-6, which in turn is able to activate macrophages and lymphocytes. CXC and CC chemokines and LTB_4 may attract and up-regulated adhesion molecules may facilitate migration of inflammatory cells to the anterior uvea. The up-regulation of adhesion molecules could be caused by endotoxin directly or by pro-inflammatory cytokines and IL-4. Interferon-γ secreted by infiltrating lymphocytes is likely to activate macrophages, which together with neutrophils under the influence of cytokines may secrete PGE_2 and NO. This could result in breakdown of the blood-aqueous barrier. Matrix metalloproteinases are known to be able to

cause major tissue destruction. Cytokines like IL-10, TGF-β_2 and even IL-12 as well as IL-1RA and FasL-induced apoptosis may be involved in the disease resolution.

The pathogenesis of EMIU appears to involve an autoimmune, delayed-type hypersensitivity immune response directed against an undefined antigenic epitope located on the anterior uveal epithelium. T cells, neutrophils and macrophages have been shown in the inflammation site. T cells are likely to use the ICAM-1/LFA-1 interaction to break the blood-aqueous barrier. Likewise in EIU, TGF-β_2 and FasL-mediated apoptosis may restrict the uveal inflammation in EMIU. In contrast to mononuclear cells neutrophils survive longer in the anterior uvea and could lead to tissue destruction by secreting TNF-α and NO.

Human AAU, EIU and EMIU have many similarities in respect to clinical picture. (Table 2) Protein flare and cells in the aqueous, miosis and posterior synechiae are typical features in all forms. Moreover, fibrin clots and hypopyon occur in severe inflammation in rodents as well as in human disease. In contrast to human disease the AU in rats is bilateral. In EIU, especially in mouse, some posterior segment involvement has been observed. Further, rats with EMIU may show signs of choroiditis following severe anterior segment inflammation. Interestingly, in 1994 Rodriguez et al. have reported of patients with severe AAU and AS/IBD whose eye inflammation turn to bilateral, affecting also posterior uveal structures and causing complications. In disease resolution and spontaneous recurrence EMIU mimics human disease compared with EIU, which remits in one week and can be re-induced only by repeated endotoxin injections. Whereas EMIU and human AAU may occur at any age, although the first attack most commonly turns up in young and middle-aged adults in the latter case, Lewis rats are resistant to EIU in older age. Characteristically HLA-B27 associated human AAU has been considered to affect more often males but severity has not been thought to be gender dependent. EIU is more severe but not more common in male rodents compared with female rodents, while in EMIU no gender-related differences have been found. In summary, from the two experimental animal models EMIU resembles more human AAU.

Table 2. Comparison of the clinical features of human acute anterior uveitis and experimental models in the rat

Clinical features	Acute anterior uveitis	Endotoxin-induced uveitis	Experimental melanin-induced uveitis
Species	human	mouse/rat	rat
Inductor	?	LPS	bovine choroidal melanin
Route of entry of antigen	mucosa ?	intravenous, intraperitoneal, intravitreal, subcutaneous	intravenous, intraperitoneal
Clinical signs			
· Ciliary injection	+	-	-
· Keratic precipitates	+	+	+
· Aqueous flare	+	+	+
· Aqueous cells	+	+	+
· Fibrin	+	+	+
· Hypopyon	+	+	+
· Hyphaema	+	+	+
· Miosis	+	+	+
· Iris hyperaemia	+	+	+
· Anterior synechiae	+	-	+
· Posterior synechiae	+	+	+
Time course	6 weeks	<1 week	4 weeks
Recurrence	Spontaneous	Induced by repeat injection	Spontaneous
Gender specificity	More common in males, but not more severe	More severe in males, but not more common	No specificity
Age specificity	More common in adults, but affects all ages	Young animals	All ages

Because of the lack of human uveitic ocular specimens available for study of the pathogenetic mechanisms of human AAU, one cannot directly translate findings from the rodent models to humans. However, the clinical similarity between the two animal models of AAU and human disease suggests the possibility that there may also be parallels at cellular and molecular levels.

4. AIMS OF THE STUDY

The aims of this study were:

1. To evaluate the role of HLA-B27 antigen in the categorization of uveitis.

2. To examine the prognosis of patients with AAU with respect to ocular and systemic complications.

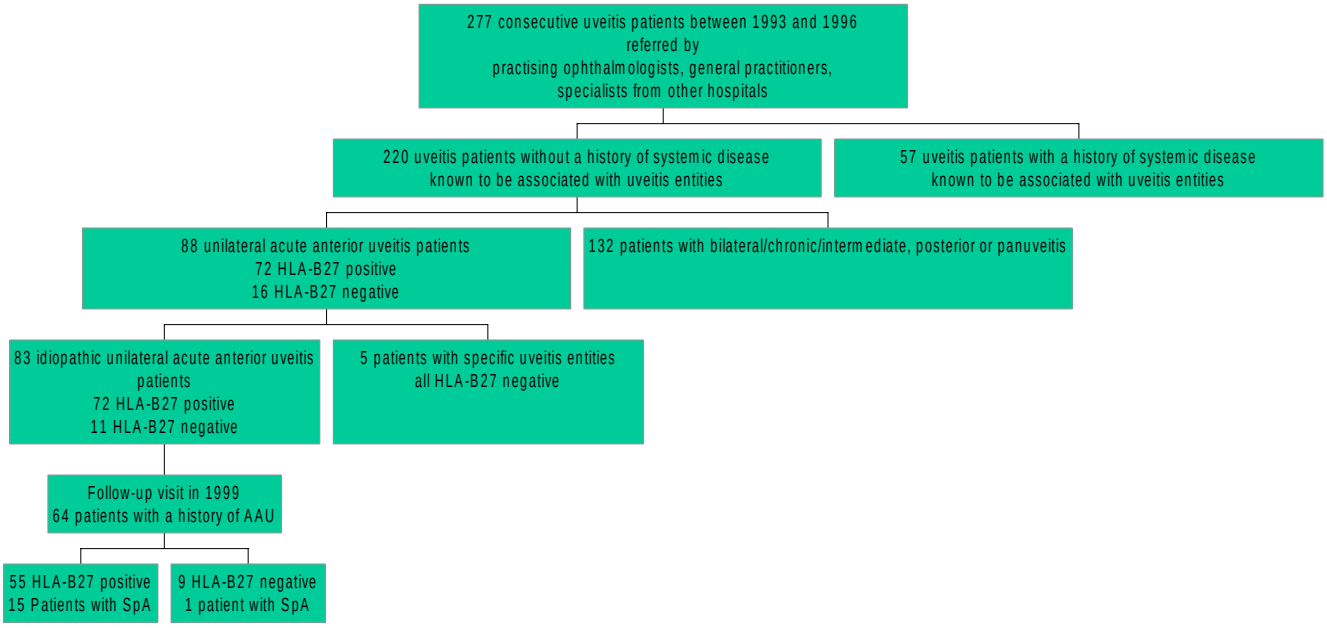
3. To explore whether infections associated with SpA, especially with ReA play a role in the etiology and outcome of AAU.

4. To investigate systemic inflammation and innate immune response in patients with previous AAU.

5. SUBJECTS AND METHODS

5.1 Patients

We conducted a prospective study of patients treated for uveitis in the outpatient clinic at the Helsinki University Eye Hospital between March 1993 and February 1996. Altogether 277 consecutive patients were referred to the clinic by practicing ophthalmologists, general practitioners or specialists from other hospitals in southern Finland. Figure 1 shows how the patients in our studies I-IV were selected.



Each patient´s history of ocular and systemic diseases and also his or her family history were recorded. Detailed questions about the occurrence of musculoskeletal, abdominal and skin diseases and respiratory, genitourinary and neurological symptoms, tick bites, and journeys abroad were asked. Data concerning age, gender, age at onset of first uveitis, number of attacks, and duration of present attack were collected on standard forms. On the basis of the clinical data, 57 patients with evidence of prior or concomitant systemic diseases known to be associated with uveitis entities were excluded from the study, leaving 220 patients with a diagnostic challenge. These 220 patients consist the population among which the categorization of uveitis by HLA-B27 typing was evaluated (I).

Eighty-eight patients were diagnosed as having unilateral AAU, five of who showed clinical picture consistent with specific uveitis entities leaving 83 patients with idiopathic AAU. They were invited to a follow-up examination. Nineteen patients did not attend: one was serving in the army, five had moved from the locality, eight refused and five did not respond to the letters or telephone calls. So, altogether 64 patients with idiopathic acute or recurrent anterior uveitis were examined again between September and December 1999 at a follow-up visit.

5.2 CONTROLS

For the second and third study 64 healthy sex- and age-matched subjects of the hospital and laboratory staff or their acquaintances served as controls in respect to the presence and levels of microbial antibodies. They did not have a history of AAU or SpA. In the fourth study for the whole blood culture assay additional 37 volunteers (26 women, 11 men, mean age 42 years, SD 10.1) were gathered and parallel blood samples were collected from the patients and from these 37 controls belonging to the hospital and laboratory staff and who were not on medication nor had any signs of infection.

5.3 OPHTHALMOLOGIC EXAMINATION

A visual acuity test, tonometry, a slit-lamp examination, and an evaluation of the fundus with a 90-dioptre lens, or indirect ophthalmoscopy, and a three mirror lens when necessary, were performed. Uveitis was graded as anterior, intermediate, posterior or panuveitis according to the criteria of the International Uveitis Study Group (Bloch-Michel and Nussenblatt, 1987).(Table 3) Uveitis was considered acute if it disappeared within three months and chronic if not. In cases of one or more previous acute attacks, it was categorized as recurrent. Furthermore, when both eyes were affected simultaneously, the uveitis was classified as bilateral; otherwise it was recorded as unilateral. In the diagnosis of non-systemic specific ocular entities, current diagnostic criteria (Nussenblatt et al., 1996) were followed. In addition to a careful slit-lamp examination each patient's history on the diagnosis of systemic disease was always confirmed by respective specialists.

Table 3. The diagnostic work-up of uveitis

Following details were taken into account:

1. onset of uveitis
2. laterality of uveitis
3. anatomic location of uveitis
4. duration of uveitis
5. granulomatous features
6. demography of the patient
7. other biomicroscopic features characteristic of known uveitis entities
8. severity of uveitis
9. general medical history
10. tailored laboratory testing

5.4 RHEUMATOLOGIC EXAMINATION

A detailed rheumatological survey with specific questions on history and clinical examination was performed on the patients with low back pain or peripheral joint symptoms suggestive of AS (diagnostic criteria included in table 4) or other forms of SpA, completed with radiology of lumbosacral spine and sacroiliac joints. Patients fulfilling the criteria of the European Spondylarthropathy Study Group (Dougados et al., 1991b) were diagnosed as having seronegative SpA.

Table 4. Modified New York criteria for ankylosing spondylitis (1984)

A. Diagnosis
1. Clinical criteria a) Presence of pain and stiffness at the dorsolumbar junction or in the lumbar spine at least 3 months duration revealing in motion, but not in rest b) Limitation of the lumbar spine both in sagittal and frontal planes c) Limited chest expansion compared to normal values of age- and sex-matched healthy subjects 2. Radiologic criteria sacro-iliitis grade $\geq$2 bilateral or grade 3-4 unilateral
B. Grading
1. Definite AS is present if the following are fulfilled: radiologic criterion with at least one clinical criterion 2. Probable AS is present if: a) 3 clinical criteria are fulfilled, or b) radiologic criterion is fulfilled without any symptoms or signs

5.5 RADIOLOGIC EXAMINATION

The radiographs were evaluated blindly by one radiologist experienced in musculoskeletal radiology. The sacroiliac findings were registered as grade 0 (normal), grade 1 (suspected), grade 2 (certain),

grade 3 (advanced), or grade 4 (beginning of bone formation) or grade 5 (ankylosis) sacroiliitis. Each sacroiliac joint was evaluated separately. The lumbar spine was graded as normal, or to show evidence of AS such as squaring or syndesmophytes (marginal, lateral) (Dale and Vinje, 1985).

5.6 LABORATORY METHODS

5.6.1 Blood samples, high-sensitivity CRP assay and routine laboratory tests

5.6.1.1 Acute phase

Between 1993 to 1996, 85% of all the patients with uveitis were tested for HLA-B27 antigen with a flow cytometer using a Becton Dickinson HLA-B27 kit (specificity 99.2%) (Bonnaud et al., 1999). Because Finland is endemic for Lyme borreliosis (Junttila et al., 1994), antibodies to *Borrelia burgdorferi* and treponemal serology were examined for 86% of the patients on the basis of the differential diagnosis. The results of these patients have been reported elsewhere (Mikkilä et al., 1997). Other laboratory and *x* ray examinations were performed if indicated by the medical history, the anatomical classification of uveitis, and the clinical and biomicroscopic specific features characteristic of uveitis associated disorders. In patients with granulomatous uveitis and /or with symptoms suggesting sarcoidosis, serum angiotensin converting enzyme and serum lysozyme were analysed, and the chest *x* rays were examined. Antitoxoplasma antibodies were tested for patients with a focal retinochoroiditis. Antinuclear antibodies were tested for children under 16 years of age with joint symptoms and for adults when a connective tissue disease was suspected.

5.6.1.2. Follow-up

In 1999 at the follow-up visit of the 64 patients with AAU parallel blood samples were obtained by phlebotomy on a given day from one to three patients and a healthy control subject. Two blood samples were collected from each subject. One was taken into a polystyrene tube (Falcon No. 2058, Becton Dickinson Labware, Lincoln Park, NJ) containing pyrogen-free heparin (10 IU/mL blood), cooled immediately at 0^0C, and aliquoted within an hour of the sampling for whole blood culture and the staining of cell-surface markers. The other was taken into a glass tube (Venoject VT-100PZX, Terumo

Europe NV, Leuven, Belgium) and similarly cooled. Serum was separated by centrifugation at 4^0C and stored in aliquots at -70^0C until use.

At the follow-up visit serum samples for antibody detection from patients with AAU were stored at -20^0C until tested simultaneously. PBMC were isolated using Vacutainer® CPT™ Cell Preparation Tube with Sodium Citrate (Becton Dickinson Vacutainer Systems, Becton Dickinson & Co., Franklin Lakes, N.J., USA) according to the manufacturer's recommendations. Within two hours of the sample collection, the tube was centrifuged (1650g, 20 min) at room temperature. After centrifugation, the cell layer containing PBMC was collected, the cells were washed once with phosphate buffered saline (PBS) and resuspended in PBS. Aliquots (100 µl) of the cell suspension were centrifuged (150g, 5 min) onto 12 microscope slides (10^5 PBMC/slide). The slides were then air dried and fixed with cold acetic acid-ethanol (5%/95% volume/volume) at –20^0C for 10 minutes, dried in air, and stored at –20^0C until stained for microbial antigens.

The immunonephelometric high-sensitivity CRP assay (Dade Behring, Marburg, Germany) was used to determine the serum CRP levels (detection limit 0.18 µg/mL) in patients with AAU. At serum CRP levels of 0.58 µg/mL and 3.55 µg/mL, the intra-assay variation was 3% and 2%, respectively, and the interassay variation was 4% and 3%, respectively.

In addition, for patients with AAU routine laboratory tests (erythrocyte sedimentation rate, blood cell count, *Treponema pallidum* hemagglutination assay, urine sediment) were performed at the control visit.

5.6.2 Antibodies

5.6.2.1 *Salmonellae, Yersiniae, Klebsiella pneumoniae, Escherichia coli* and *Proteus mirabilis*

Yersinia enterocolitica O:3 and *O:9* and *Y. pseudotuberculosis* I and III were clinical isolates from the Department of Medical Microbiology, University of Turku (Turku, Finland). The antigen extracts were prepared as previously described (Granfors et al., 1989b). Antibodies against *Salmonella* were studied using combined lipopolysaccharides from *S. enteritidis* and *S. typhimurium* (Sigma Chemical Co., St. Louis, MO, USA) as antigen (Isomäki et al., 1989). *S. enteritidis*, *S. typhimurium* and other *Salmonellae* belonging to groups B or D are responsible for ~90% of Salmonella infections diagnosed

by bacterial isolation in Finland. The remaining *Salmonella* subtypes are probably also recognized by this ELISA, although with less efficiency (Isomäki et al., 1989).

Klebsiella pneumoniae strains 21, 43 and ATCC 27736, with the capsular antigens 21, 43 and 30, respectively, were chosen on the basis of previous studies in which the strains were thoroughly studied for their role in the pathogenesis of AS and other SpAs (Nissilä et al., 1994; Mäki-Ikola et al., 1997; Höhler et al., 1999). *K. pneumoniae* strains 21 and 43 were provided by Dr AF Geczy (New South Wales Red Cross Blood Transfusion Service, Sydney, Australia), and strain ATCC 27736 came from the American Type Culture Collection (Rockville, MD, USA). *E. coli* and *Proteus mirabilis* were clinical isolates from the Department of Medical Microbiology, University of Turku, Turku, Finland. The antigen extracts were prepared as previously described (Granfors et al., 1989b).

The polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with sodium dodecyl sulphate (SDS) extracts of *Y. enterocolitica* O:3, O:9, *Y. pseudotuberculosis I* and *III* (0.5 μg/ml), *K. pneumoniae* 21 and 43 (1 μg/ml), ATCC 27736 (5 μg/ml), *E. coli* (0.5 μg/ml) and *P. mirabilis* (2 μg/ml) and with lipopolysaccharides of *S. enteritidis* and *S. typhimurium* in PBS overnight at 37^0C. The plates were thereafter saturated with 1% bovine serum albumin (BSA-PBS). Serum samples, diluted 1:250, were incubated on the plates for two hours at 37^0C. Thereafter alkaline-phosphatase-conjugated swine anti-human IgM or IgA (Orion Diagnostica, Espoo, Finland, diluted 1:250 or rabbit anti-human IgG (Dako, Glostrup, Denmark), diluted 1:2500, were incubated on the plates overnight at room temperature. Fresh p-nitrophenyl phosphate in diethanolamine-magnesium chloride buffer (1 mg/ml) (Oy Reagena Ltd, Kuopio, Finland) was added and incubated for 30 minutes at 37^0C. The reaction was stopped with 1M sodium hydroxide. The optical density was measured with VICTOR^{TM} 1420 Multilabel Counter (Wallac, Turku, Finland) at a wavelength of 405 nm.

Antibody concentrations are expressed as enzyme immunoassay (EIA) units where one EIA unit is 1/100 of the corresponding antibody concentration in the positive reference serum. Antibody titers that are at least two standard deviations above the mean of healthy control persons were regarded as positive.

5.6.2.2 *Campylobacter jejuni*

The antigen for the ELISA measurement of antibodies to *Campylobacter* was an acid extract of *Campylobacter jejuni* strain 143483 prepared and used as earlier described (Rautelin and Kosunen, 1983). Dilution series of sera in 0.05% Tween 20-0.5% gelatin-PBS buffer were applied to the plates and incubated overnight. With intervening washes, the following reagents were applied: biotin labeled

anti-human IgG, IgA, or IgM prepared in goats, alkaline phosphatase labeled streptavidin (each at 1/5000 dilution, Zymed, California, USA), 4-nitrophenyl phosphate substrate in 0.02 M diethanolamine buffer - 1 mM Mg^{2+} pH 10.0. The end-point titers were generated from the measured OD405 values and evaluated with the aid of local standards. A titer level of 3500 or more for IgG, 5000 for IgA, and 2500 for IgM was considered to indicate previous infection due to *Campylobacter jejuni*.

5.6.2.3 *Chlamydia pneumoniae* and *Chlamydia trachomatis*

Antibodies specific for *C. pneumoniae* were measured by the microimmunofluorescence (MIF) (Wang et al., 1986) test using purified elementary bodies of the Finnish epidemic isolate Kajaani-6 as antigen. Sera were tested in serial 4-fold dilutions from 1:32 for immunoglobulin (Ig) G antibodies and screened for IgM and IgA antibodies in a 1:16 dilution with fluorescein-isothiocyanate-conjugated antihuman Ig. All the initially IgM- and IgA-positive serum samples were absorbed with IgG removal reagent (Gullsorb, Gull Laboratories, Salt Lake City, UT, USA) and retested in the MIF assay in serial dilutions. An antibody titer of ≥1:512 in the IgG fraction, ≥1:16 in the IgM fraction or ≥1:160 in the IgA fraction was considered to be an indicator of an ongoing infection from *C. pneumoniae*, and a titer of 1:32-1:256 for IgG, ≥1:16 for IgM, and ≥1:16 for IgA was considered to indicate previous infection due to *C. pneumoniae*. *C. trachomatis* antibodies were also studied by MIF (Wang et al., 1975). The cut-off points for ongoing or previous infection from *C. trachomatis* were the same as for *C. pneumoniae*.

5.6.2.4 *Chlamydia pneumoniae* Hsp60 and human Hsp60

Antibodies to *C. pneumoniae* and human Hsp60 were measured by an enzyme immunoassay (EIA). Polystyrene 96-well plates (Nalge Ltd., Hereford, United Kingdom) were coated with recombinant Cpn Hsp60, produced in *Bacillus subtilis* (Airaksinen et al., 1998) (5 μg/ml) with C-terminal His_6-tag and human Hsp60 (Sigma, St. Louis, MO, USA) overnight at room temperature. Residual binding was blocked by incubation with 3% bovine serum albumin (BSA). Sera diluted 1:100 in phosphate-buffered saline containing 1% BSA were allowed to bind to the wells. The plates were washed, and the binding was detected with horseradish-peroxidase-labeled antibody to human IgG (Cpn Hsp60) and IgA (Cpn Hsp60, human Hsp60) (Dako A/S, Denmark). After the washing, the substrate (BM Blue POD substrate, Boehringer Mannheim, Germany) was added, and the absorbance was measured at 450 nm. The results were expressed as EIA units, calculated by multiplying the optical densities by 100. Values

above the mean plus 2 standard deviations (SD) of the controls (≥19.7 EIA units) were considered suggestive of previous exposure to *C. pneumoniae*.

5.6.2.5 *Borrelia burgdorferi*

IgG and IgM antibodies to *B. burgdorferi* were measured by indirect ELISA with purified 41 kDA flagellin as the antigen (Dako kit, Glostrup, Denmark, Lyme ELISA, modified by endpoint titration) (Seppälä et al., 1994).

5.6.3 Detection of microbial antigens in peripheral blood mononuclear cells

To study the presence of *Salmonella* or *Yersinia* antigens in the cells, the slides of each patient PBMC stored at -20^{0}C were overlaid with mouse monoclonal antibodies specific for O-polysaccharide chains of lipopolysaccharides of *S. enteritidis* (MASE O9) (Granfors et al., 1990), *S. typhimurium* (MAST O4) (Granfors et al., 1990), *Y. enterocolitica* O:3 (A6) (Pekkola-Heino, 1987; Granfors et al., 1989a; Granfors et al., 1998), and the heat-shock protein of *Y. enterocolitica* O:3 (IV7D2) (Granfors et al., 1998; Probst et al., 1993). Subclass-matched monoclonal antibody 3G6, which is specific for chicken T cells, was used as a negative control.
The reactivity and specificity of antibodies was ascertained with this technique as described earlier (Granfors et al., 1989a; Granfors et al., 1990; Granfors et al., 1998). The slides were incubated at room temperature for 30 minutes and washed three times with BSA-PBS. They were then stained with fluorescein-labeled F(ab')$_2$ fragments of anti-mouse IgG (1:200; Sigma Chemical Company, St. Louis, MO) at room temperature for 30 minutes, washed again, dried and mounted in PBS/glycerol (1:9, v/v), which contained 1 mg/ml p-phenylenediamine (Sigma), and were analyzed using a fluorescence microscope (Leitz diaplan-incidence light fluorescence microscope with an Osram HBO 100-watt mercury lamp (Leitz, Wetzzlar, Germany). All the slides were read by an experienced reader, who was blinded as to the source of the cells.

C. pneumoniae DNA was detected in PBMC as described by Boman et al (Boman et al., 1998) with slight modifications. In brief, PBMC were isolated from an 8-ml blood sample collected in Vacutainer CPT (Beckton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. DNA was extracted from the PBMC fraction with a QIAamp DNA kit (Qiagen, Valencia, CA, USA) and stored at –70^{0}C for polymerase chain reaction (PCR). Mock extractions were always included in the sample preparation.

The extracted DNA was amplified by *C. pneumoniae*-specific nested primers in a touchdown PCR as described by Tong and Sillis (Tong and Sillis, 1993). Amplified products were detected by agarose gel electrophoresis and ethidium bromide staining, as well as by Southern blotting and hybridization with a digoxigenin-labeled probe. Positive and negative controls were included in each run. Designated areas were used for the sample preparation, PCR amplification and product analysis to avoid contamination of the specimens.

5.6.4 HLA-B27 typing

In the follow-up visit in 1999 the presence of HLA-B27 antigen in the 64 patients had been determined previously using the standard microlymphocytotoxity technique. In HLA-B27 negative patients the result was ascertained and in healthy control subjects the presence of HLA-B27 gene was studied using PCR method of the diagnostic molecular genetics laboratory of the hospital. In brief, DNA was amplified with primers B27/E136 as 5'-CGG CGG TCC AGG AGC T-3'5'- and B27/E91 s +5'-GGG TCT CAC ACC CTC CAG AAT-3'. Generation of the HLA-B27 PCR product was detected on agarose gel electrophoresis.

5.6.5 Production of TNF-α

The reagents used were pyrogen-free heparin (Lövens, Ballerup, Denmark); Dulbecco's phosphate buffered saline (PBS) and RPMI 1640 medium (both from Life Technologies Ltd., Paisley, United Kingdom); *Escherichia coli* O111:B4 lipopolysaccharide (LPS, Zigma, St. Louis, MO); TNF-α Sample Diluent (Diagnostic Products, Los Angeles, CA); fluorescein isothiocyanate (FITC) conjugate of mouse anti-CD11b mAb (IgG1, clone BEAR 1), phycoerythrin (PE)-CY5 (PC5) conjugate of anti-CD16 mAb (IgG1, clone 3G8), and PC5 conjugate of irrelevant mouse mAb IgG1 (clone 679.1Mc7) (all from Immunotech, Marseille, France); RPE conjugate of anti-CD14 mAb (IgG2a, clone TÜK4) and RPE conjugate of irrelevant mAb (IgG2a, clone DAK-GO5) (both from DAKO A/S, Glostrup, Denmark); and FACS lysing solution (Becton Dickinson, San Jose, CA). QuantiBRITE PE bead standards were obtained from Becton Dickinson.

LPS stock solution (400 mg/mL in PBS) was stored in 20-μL aliquots at -20^{0}C. Samples of 100 μL of heparinized blood were added to the polypropylene tubes (No. 352063, Becton Dickinson) containing 800 μL of RPMI 1640 and 100 μL of LPS (final concentrations 1000 ng/mL and 10 ng/mL) in PBS, or PBS only. After incubation for 4 h at 37^{0}C in 5% CO_2, the supernatants were separated by centrifugation, snap-frozen, and stored at -70^{0}C. The TNF-α levels were determined within two weeks. The analysis of the time course of TNF-α production indicated that, in agreement with previous studies

of whole blood (Desch et al., 1989; Wilson et al., 1991; Nerad 1992), near maximal TNF-α levels of the culture supernatant were obtained during the 4-h incubation period (data not shown). The intra-assay variation was 9% and 7% for LPS 1000 ng/mL and 10 ng/mL, respectively, and the interassay variation was 12% for both concentrations.

The culture supernatants at -70^{0}C were thawed and diluted 1:5 with TNF-α Sample Diluent. The TNF-α levels of the culture supernatants were determined by Immulite (Diagnostic Products Company, Los Angeles, CA), a chemiluminescent immunoassay system. The detection limit of TNF-α was 4 pg/mL. The TNF-α levels were corrected for dilution.

5.6.6 Determination of sIL-2R

The sIL-2R levels of the serum samples were determined by Immulite (Diagnostic Products Company, Los Angeles, CA). The detection limit of sIL-2R was 10 U/mL.

5.6.7 Flow cytometry

Three-color flow cytometry was used. The cell labeling was as previously described (Repo et al., 1993). Briefly, two 25-µl aliquots of heparinized whole blood in polystyrene tubes (No. 352054, Becton Dickinson) were triple-labeled at 0^{0}C by the addition of pretitrated amounts of FITC-conjugated CD11b mAb to both tubes, PE-conjugated CD14 mAb (tube 1) or irrelevant PE-conjugated IgG2a mAb (tube 2), and PC5-conjugated CD16 mAb (tube 1) or irrelevant PC5-conjugated IgG1 mAb (tube 2). After being stained for 20 min at 0^{0}C, erythrocytes were lysed by the addition of 1:10-diluted ice-cold FACS lysing solution. After a 3-min incubation at a cold temperature, the cells were separated by centrifugation and resuspended in 2 mL of FACS lysing solution at room temperature. Leukocytes were pelleted by centrifugation and resuspended in 1% formalin at 0^{0}C. Data were acquired by a flow cytometer within six hours.

A FACSort flow cytometer (Becton Dickinson) and CellQuest software were used to obtain the data. After appropriate spectral compensations, the instrument settings were not changed during the study. An electronic live gate for CD11b-positive monocytes was set as follows: first, the CD11b-positive events were delineated in the side scatter (SSC)/CD11b(fluorescence-1 axis, FL1) dot plot by the region R1 (**IV**/Fig. 1A); second, an SSC/forward scatter (FSC) dot plot (**IV**/Fig. 1B) was created from R1, and R2 was set to delineate the monocyte population. At least 1x10^3 monocytes (i.e., events colocating in R1 and R2) were collected. The QuantiBRITE PE bead standards (i.e., beads conjugated with four known levels of PE) were run weekly during the study period.

The data were analyzed using QuantiCalc software (Verity Software House, Topsham, ME). The QuantiBRITE PE data were used to calibrate the FL2 axis in terms of PE molecules. With the use of the CD14(FL2)/CD16(FL3) dot plot (**IV**/Fig.1C), $CD14^{bright}CD16^{-}$ and $CD14^{dim}CD16^{+}$ monocyte subsets were identified. According to the manufacturer, the ratio of fluorochrome to protein is 1 in the RPE conjugate of the CD14 mAb used. CD14 expression was reported as the antibody binding capacity (ABC) (i.e., the median number of PE molecules bound by the monocyte). To assess the CD11b expression, a CD11b fluorescence (FL1) histogram was created from the monocyte population, and the CD11b fluorescence intensities were reported as relative fluorescence units (RFU) (i.e., median channel number of the fluorescent cell population).

The monocyte count was determined using a flow cytometer as a cell counter, as described previously (Repo et al., 1997). The time needed to collect data from $1x10^3$ monocytes was recorded. The arithmetic mean of the monocyte count of tubes 1 and 2 was determined.

5.7 STATISTICAL METHODS

Variables within two groups normally distributed and with equal variance were analyzed using the equal-variance t test and those with a non-normal distribution using the Mann-Whitney U-test. Categorical data were analyzed by the Pearson chi-square test or Fisher's exact test. To estimate dependencies between presence of antibodies and the patients' characteristics we used a logistic-regression model. Clinically important variables were chosen for the analysis. The forward selection method was used and cut off values were set at 0.2 for the variable selection.

In the third original communication proportional data were compared with the geometrical mean test (GMT), the paired comparison was carried out with the Wilcoxon signed ranks test. No adjustment was made for multiple testing.

Out of the 64 patients included in the study IV, five with ongoing recurrent attack of AAU and three others with incomplete data in the culture assay and cell surface marker study were excluded from the data analyses of the fourth original communication. The results are presented as median values with interquartile range (IQR). The patient and control groups were compared with the Mann-Whitney U test. The relationship between the monocyte count and TNF-α production was analyzed with the Spearman rank correlation coefficient (r) and its 95% confidence interval (CI), and with a locally weighted scatter plot smoother (LOWESS). The α level was 0.05 for all the statistical tests.

6. RESULTS

Roman numerals (**I-IV**) refer to the original communications.

6.1 HLA-B27 DISTRIBUTION, CLINICAL CHARACTERISTICS AND OUTCOME OF THE PATIENTS

6.1.1 Acute phase

Two hundred and twenty patients (99 men and 121 women) had no established systemic disease known to be associated with uveitis entities when they entered this study (Fig 1). At the time of the first symptomatic uveitis attack the subjects´ median age was 36 (range 9-91) years.

Seventy-one per cent of the patients with anterior uveitis but only 7% of the patients with intermediate, posterior or panuveitis were positive for HLA-B27 antigen (χ^2, $p<0.0001$) (Table 5).

Table 5 HLA-B27 antigen and anatomical location of uveitis

HLA-B27 antigen	Anterior (n=112)	Intermediate (n=36)	Posterior (n=31)	Panuveitis (n=41)
positive	75	1	1	4
negative	30	33	14	28

In addition 79% of the patients with acute or recurrent unilateral disease, but only 7-12% of those with chronic and/or bilateral disease were HLA-B27 positive (χ^2, $p<0.0001$)(Table 6).

Table 6 HLA-B27 antigen, and duration and laterality of uveitis

Classification	No. of patients tested for HLA-B27/ total no.	No. of HLA-B27 positive in tested (%)
Acute / recurrent unilateral	92 / 111	73 (79)
Acute/ recurrent bilateral	17 / 18	2 (12)
Chronic unilateral	30 / 36	2 (7)
Chronic bilateral	47 / 55	4 (9)

Of the patients with unilateral acute or recurrent anterior uveitis, 72 (82%) were HLA-B27 positive and 16 (18%) were HLA-B27 negative (Table 7). In the latter group, biomicroscopic specific features gave clues to distinct uveitis entities in five cases. Three patients had patchy or sectorial iris atrophy combined with fatty keratic precipitates suggestive of herpetic infections, one patient had sarcoid lung manifestations in the chest *x* ray and histologically confirmed conjunctival granulomas, and one patient had a clinical picture consistent with Posner Schlossman uveitis. Of the 83 idiopathic unilateral AAU patients in our study, 13% (7 male, 2 females) were diagnosed as having SpA. There were 27 patients with SpA that had been diagnosed earlier in the excluded group of 57 patients, five of whom had posterior eye involvement (Fig 1).

Table 7. General characteristics and biomicroscopic features in unilateral AAU cases

General characteristics and biomicroscopic features	Idiopathic B27+ (n=63)	AS SpA* B27+ (n=9)	Idiopathic B27- (n=11)	Other uveitis entity B27- (n=5)
Median age at onset (years)	31	28	45	41
Male:female	1,3:1	3,5:1	1,2:1	2:3
Recurrent (%)	49	78	27	40
Fibrin exudate (%)	43	56	55	60
Hypopyon (%)	11	0	18	0
Fatty keratic precipitates (%)	6	0	0	75
Patchy/sectorial iris atrophy	0	0	0	60
Persistent post. synechiae (%)	22	22	18	40

* Ankylosing spondylitis or other seronegative spondyloarthropathy

In the entire group of 220 patients, HLA-B27 positivity was unevenly distributed among the patients with different uveitis entities or associated disorders.(Table 8) The number of different uveitis entities followed the same pattern like in other published reports from tertiary eye care centres. Interestingly, if idiopathic or SpA related AAU cases were excluded, HLA-B27 existed less often in patients with other uveitis entities (7.8% in tested) compared to the general population (14%) in Finland.

Table 8. Distribution of HLA-B27 antigen in uveitis entities or associated disorders

Uveitis entity or associated disorder	No. of patients tested for HLA-B27 / total no. (%)	No. of HLA-B27 positive in tested (%)
Idiopathic AAU (unilateral)	74 / 74 (100)	63 (85)
AS/SpA *	10 / 10 (100)	10 (100)
Unknown	42 / 48 (88)	4 (10)
Infection **	18 / 30 (60)	2 (11)
Other systemic disease ***	8 / 14 (57)	1 (13)
Specific ocular entity ****	34 / 44 (77)	1 (3)
Total	186 / 220 (85)	81 (44)

* Akylosing spondylitis 2, undetermined spondyloarthropathy 8 (7 unilateral acute anterior, 1 bilateral chronic anterior)
** Toxoplasmosis 13, herpetic infections 8, Lyme borreliosis 7, cytomegalovirus infection 1, streptococcal infection 1
*** Sarcoidosis 12, chronic lymphatic leukemia 1, lupus erythematosus disseminatus 1
**** Fuchs´ heterochromic iridocyclitis 12, pars planitis 9, white dot syndrome 8, idiopathic retinal vasculitis 8, Posner Schlossman syndrome 2, ischemic uveitis 2, birdshot retinochoroidopathy 2, infectious bilateral papillitis 1

The median age at the time of the first uveitis attack was significantly lower (30.5 versus 45 years) (Mann-Whitney U test, p=0.007) for the HLA-B27 positive unilateral AAU group (**I**/Fig 1) than for the group with HLA-B27 negative idiopathic unilateral AAU (**I**/Fig 2). The male-to-female ratio was 1.2:1 and 1.3:1 for the idiopathic HLA-B27 positive and negative groups, respectively (Table 8). As suspected the male dominance (3.5:1) was more evident among the patients with AS or other SpAs than among the idiopathic cases. However, in the age group of over 40 years, there was a tendency towards female dominance among the HLA-B27 positive unilateral AAU cases (**I**/Fig 1).

Patients with HLA-B27 positive unilateral AAU, especially when associated with AS or other SpAs, had a history of recurrent attacks more often than patients with idiopathic HLA-B27 negative unilateral AAU (Table 8). The presence of fibrin exudate was not observed to be more frequent in patients with idiopathic HLA-B27 positive unilateral AAU (43%) than in those with idiopathic HLA-B27 negative unilateral AAU (55%) or in those with AS or other SpAs (56%). Hypopyon was detected in 11% of the HLA-B27 positive and 18% of the HLA-B27 negative patients.

6.1.2 Follow-up

At the follow-up visit in 1999 of the patients with unilateral AAU there were 64 (38 men and 26 women) left, with a mean age of 45.4 (SD 12.8) years (Fig 1). A mutual amount of sex- and age-matched healthy control subjects gave parallel blood samples. Because the presence of HLA-B27 antigen in our patients had been determined previously using the standard microlymphocytotoxity technique, we wanted to ascertain the result and study the presence of HLA-B27 gene using PCR method in HLA-B27 negative patients and in healthy control subjects. The HLA-B27 negative patients

proved to be negative also by PCR method. Fifty-five (86%) of the patients and six (9%) of the control subjects were HLA-B27 positive.

Four-fifths (84%) of the patients had had a recurrent attack. The mean age at the onset of the first AAU was 34.2 (SD 11.6, range 14-64) years, and time between the first attack and the follow-up visit was 11.1 (SD 9.6) years. Four male patients and one female patient had recurrent uveitis attack at the time of the follow-up examination. None of the patients had symptoms of clinical infection within six months before the recruitment or antibody levels diagnostic for recent infection.

By the time of the follow-up visit out of the 64 patients altogether 25%, 11 male (one male with SpA from the cohort of 83 patients had dropped out) and 5 females, had developed SpA, and 27% had been affected by eye complications (i.e., persistent synechiae, cataract, cystoid macular degeneration and posterior or panuveitis). In addition, seven patients had more than one complication per eye, and three patients had complications in both eyes. In 11% of the patients anterior uveitis had became chronic. The ocular complications did not associate with the presence of SpA and HLA-B27 (Table 9).

Table 9. Characteristics of the 64 patients with previous acute anterior uveitis (AAU) at the time of the follow-up examination

Characteristics	No. of patients	%
HLA-B27 antigen positivity	55	86
Recurrences of AAU		
None	10	16
Ten or less	32	50
More than ten	22	34
Chronic eye disease*	7	11
Eye complications	17	27
Cataract†	9	7
Cystoid macular degeneration†	5	4
Glaucoma†	0	0
Persistent synechiae†	13	10
Posterior uveitis or panuveitis†	2	2
More than one complication per eye	7	11
Complications in both eyes	3	5
Spondyloarthropathy	16	25

*Iritis not resolved within three months

†Data are number of eyes affected with complications

6.2 INFECTIOUS BACKROUND

The prevalences of the antibodies to Y. *enterocolitica and Y. pseudotuberculosis III*, *Salmonellae, K. pneumoniae*, *P. mirabilis*, *E. coli*, *Chlamydia* and *Borrelia* species for the patients and the control subjects were much the same (Table 10). Patients with AAU tended to have antibodies to *Yersinia pseudotuberculosis I* more often than the control subjects (28% vs. 11%, p=0.01; Table 10), but if corrected with the number of comparisons performed, the significance disappeared. The prevalence of IgA ($p<0.0001$) as well as IgG antibodies (p=0.02) to *C. pneumoniae* was detected more often in controls than in patients (Table 10).

Table 10. Prevalence of antibodies in the patients (n=64) and the control subjects (n=64).

Prevalence of antibodies	**Patients**		**Controls**	
	No.	**(%)**	**No.**	**(%)**
Salmonellae				
IgA	4	(6.3)	5	(7.8)
IgG	2	(3.1)	2	(3.1)
IgM	5	(7.8)	3	(4.7)
Total positive	**10**	**(15.6)**	**8**	**(12.5)**
Yersinia enterocolitica 0:3				
IgA	2	(3.1)	2	(3.1)
IgG	3	(4.7)	3	(4.7)
IgM	1	(1.6)	5	(7.8)
Total positive	**6**	**(9.4)**	**9**	**(14.1)**
Yersinia enterocolitica 0:9				
IgA	0	(0.0)	2	(3.1)
IgG	2	(3.1)	4	(6.3)
IgM	3	(4.7)	4	(6.3)
Total positive	**5**	**(7.8)**	**8**	**(12.5)**
Yersinia pseudotuberculosis I				
IgA	3	(4.7)	3	(4.7)
IgG	6	(9.4)	3	(4.7)
IgM	10	(15.6)	2	(3.1)
Total positive	**18**	**(28.1)***	**7**	**(10.9)**
Yersinia pseudotuberculosis III				
IgA	4	(6.3)	4	(6.3)
IgG	1	(1.6)	4	(6.3)
IgM	4	(6.3)	3	(4.7)
Total positive	**9**	**(14.1)**	**9**	**(14.1)**

Prevalence of antibodies		Patients		Controls	
		No.	**(%)**	**No.**	**(%)**
Klebsiella pneumoniae strain 43	IgA	10	(15.6)	3	(4.7)
	IgG	3	(4.7)	4	(6.3)
	IgM	5	(7.8)	4	(6.3)
Total positive		**14**	**(21.9)**	**9**	**(14.1)**
Klebsiella pneumoniae strain 21	IgA	2	(3.1)	3	(4.7)
	IgG	3	(4.7)	3	(4.7)
	IgM	2	(3.1)	3	(4.7)
Total positive		**5**	**(7.8)**	**8**	**(12.5)**
Klebsiella pneumoniae strain ATCC27736					
	IgA	6	(9.4)	4	(6.3)
	IgG	1	(1.6)	3	(4.7)
	IgM	1	(1.6)	2	(3.1)
Total positive		**7**	**(10.9)**	**7**	**(10.9)**
Campylobacter jejuni					
	IgA	0	(0.0)	0	(0.0)
	IgG	4	(6.3)	2	(3.1)
	IgM	0	(0.0)	0	(0.0)
Total positive		**4**	**(6.3)**	**2**	**(3.1)**
Proteus mirabilis					
	IgA	5	(7.8)	3	(4.7)
	IgG	1	(1.6)	3	(4.7)
	IgM	5	(7.8)	3	(4.7)
Total positive		**9**	**(14.1)**	**8**	**(12.5)**
Escherichia coli					
	IgA	3	(4.7)	5	(7.8)
	IgG	5	(7.8)	2	(3.1)
	IgM	2	(3.1)	6	(9.4)
Total positive		**6**	**(9.4)**	**13**	**(20.3)**

Prevalence of antibodies	**Patients**		**Controls**	
	No.	**(%)**	**No.**	**(%)**
Chlamydia trachomatis				
IgA	1	(1.6)	0	(0.0)
IgG	3	(4.7)	4	(6.3)
IgM	0	(0.0)	0	(0.0)
Total positive	**3**	**(4.7)**	**4**	**(6.3)**
Chlamydia pneumoniae				
IgA	2	(3.1)	22	(34.4)
IgG	36	(56.3)	46	(71.9)
IgM	2	(3.1)	0	(0.0)
Total positive	**38**	**(59.4)**	**46**	**(71.9)**
Borrelia burgdorferi				
IgG	0	(0.0)	1	(1.6)
IgM	0	(0.0)	1	(1.6)
Total positive	**0**	**(0.0)**	**2**	**(3.1)**

*Pearson Chi-Square test, p = 0.01

The antibody levels against enterobacteria (i.e., *Yersiniae*, *K. pneumoniae*, *C. jejuni*, *E. coli* and *P. mirabilis*), and *C. trachomatis* as well as *B. Burgdorferi*, did not differ between the patients and the control subjects. An exception was the higher levels of IgG antibodies to S*almonellae* found in the controls (p=0.018). Elevated IgM antibodies to *Y. pseudotuberculosis I* were measured more often in the patients than controls (p=0.03). If corrected with number of comparisons made, the statistical significance could not be confirmed. Higher levels of IgA (p<0.0001) and IgG antibodies (p=0.002) to *C. pneumoniae* were detected in patients compared with controls.

Sex, age at examination, eye complications, development of SpA, recurrences or time since the first AAU or chronic course of the disease did not correlate with antibody positivity (Table 11). When a detailed analysis was made, the patients with eye complications had higher levels of IgA antibodies to *P. mirabilis* (p=0.003; if corrected for the numbers of comparisons made, p=0.042) than those without.

Table 11. Presence of antibodies in relation to clinical characteristics of the patients.

Characteristics		Patients without antibodies No.	Patients with antibodies* No.	(%)
Sex	Male	5	33	(86.8)
	Female	3	23	(88.5)
Age at examination				
	≤40 years	4	22	(84.6)
	>40 years	4	34	(89.5)
Years from the first AAU				
	≤10 years	6	39	(86.7)
	>10 years	2	17	(89.5)
Eye complications				
	None	4	43	(91.5)
	Yes	4	13	(76.5)
Spondyloarthropathy				
	None	7	41	(85.4)
	Yes	1	15	(93.8)
HLA-B27 antigen				
	Negative	2	7	(77.8)
	Positive	6	49	(89.1)
Chronic eye disease†				
	No	7	50	(87.8)
	Yes	1	6	(85.7)

* Prevalence of antibodies to individual bacteria is presented in Table 11.
†Iritis not resolved within three months

In the logistic-regression analysis to explore whether a dependency between positive antibody levels (≥4 SD of controls for *Enterobactericae* and cut off values as mentioned in methods section for *Campylobacter*, *Borrelia* and *Chlamydia* species) and the patients' characteristics existed, the independent variables were sex, age at onset of AAU, ocular complications, SpAs, time since the first AAU and number of recurrences (≤10 recurrences vs. >10 recurrences). The number of the recurrences was the only variable of statistical significance in relation to presence of antibodies against one or several bacteria (p=0.047). Although every sixth patient had serological scars from *Salmonella* and every tenth from *Yersinia enterocolitica* infection, and in addition, at least two patients were known to have had *Yersinia enterocolitica* infection 15-20 years ago at the time of the first AAU attack or during first recurrences, none of the patients had persisting *Yersinia* or *Salmonella* antigens in their PBMCs.

IgA antibodies to Cpn Hsp60 were found more often (39% vs 3%) and in higher levels (median EIA units 18 vs 10) in patients with AAU than in the controls (Wilcoxon signed ranks test, 2-tailed, p = 0.0001) (**III**/Fig. 1). However, no statistically significant difference was observed between the patients and controls in the occurrence of IgG antibodies to Cpn Hsp60. The median EIA unit of IgG antibodies was 65 for the patients and 48 for the controls (**III**/Fig. 2). *C. pneumoniae* DNA was detected in one of the patients who was not positive for *C. pneumoniae* antibodies. All the controls were negative for *C. pneumoniae* DNA.

Number of recurrences, presence of fibrin exudates, chronic course of the disease and AS or other SpAs were evenly distributed among the patients with a positive and negative titer of IgA antibodies to Cpn Hsp60. In contrast, ocular complications, (46% vs 15%) (Mann-Whitney U-test, 2-tailed, p = 0.007) were observed more often in the former group (Table 12).

Table 12. Demographics, HLA-B27 antigen, ocular features and spondylarthropathies (SpA) of 64 patients with previous acute anterior uveitis according to the distribution of immunoglobulin (Ig) A antibodies to *Chlamydia pneumoniae* Hsp60.

	Patients with positive (≥19,7 EIA* units) IgA titer (n=24)	Patients with negative (<19,7 EIA units) IgA titer (n=40)	Significance
Male:female (ratio)	3:1	1:1	ns
Age of onset, years, mean (SD)	35.8 (10.6)	3.3 (12.2)	ns
Age of examination, years, mean (SD)	47.7 (11.7)	44.0 (13.4)	ns
HLA-B27 positive (%)	83	88	ns
Recurrences (%)	75	90	ns
Occurrences (N) of iritis per year (range)	0.72 (0.11-2.5)	1.23 (0.17-5)	ns
Fibrin exudates (%)	58	60	ns
Chronic disease† (%)	17	8	ns
Eye complications (%)	46	15	p=0.007
SpA (%)	13	33	p=0.076

*Enzyme immunoassay
†Iritis not resolved within 3 months

Both eyes were affected with complications equally often in groups of patients with positive and negative levels of IgA antibodies to Cpn Hsp60. Further, no differences could be found in the existence of persistent synechiae between afore mentioned groups. Cataract, cystoid macular degeneration and posterior eye involvement, as well as a higher complication rate per eye, were observed more frequently in the group of patients with positive levels of IgA antibodies to Cpn Hsp60, although the sample size was too small for statistical conclusions to be drawn (Table 13).

Table13. Eye complications of the patients with a positive (n=11) or negative (n=6) titer of immunoglobulin (Ig) A antibodies to *Chlamydia pneumoniae* Hsp60.

	Positive IgA titer (≥19,7 EIA* units)	Negative IgA titer (<19,7 EIA units)
Complications in both eyes	2/11 patients (18%)	1/6 patients (16%)
Persistent synechiae	9/22 eyes (41%)	4/12 eyes (33%)
Cataract	7/22 eyes (32%)	2/12 eyes (17%)
CMD†	4/22 eyes (18%)	1/12 eyes (8%)
Posterior uveitis or panuveitis	2/22 eyes (17%)	0/12 eyes (0%)
>1 complication per eye	7/11 patients (64%)	0/6 patients (0%)

*Enzyme immunoassay
†Cystoid macular degeneration

In our study, HLA-B27 antigen did not correlate with antibody positivity against *Enterobactericae*, *Chlamydia* or *Borrelia* species (Table 11). Further, the HLA-B27 antigen was evenly distributed among the patients with a positive and negative titer of IgA antibodies to Cpn Hsp60 (Table 12). The HLA-B27 positive controls could not be distinguished from the HLA-B27 negative ones in terms of the levels of IgA antibodies to Cpn Hsp60 (Mann-Whitney U-test, 2-tailed, p = 0.863). Fifteen of 24 patients (63%) with positive (≥19.7 EIA units) levels of IgA antibodies to Cpn Hsp60 had serological evidence of previous infection with *C. pneumoniae*. When tested for antibodies against human Hsp60, neither the patients nor the controls had any marked levels of IgA antibodies.

6.3 SYSTEMIC INFLAMMATION

6.3.1 Production of TNF-α

Our measurements of the TNF-α levels of the whole blood culture media (LPS 10 ng/mL) correlated positively with the blood monocyte counts (**IV**/Fig. 2A); however, when standardized by the monocyte count, this correlation disappeared (**IV**/Fig. 2B).

The median TNF-α concentration (**IV**/Fig. 3) was significantly higher in the patient group than in the control group [1473 pg/mL (1193 to 2024) vs 1320 pg/mL (935 to 1555); p=0.012] under conditions in which monocyte activation is mediated specifically via high-affinity CD14 receptors for complexes of LPS and serum LPS binding protein (Ulevitch and Tobias, 1999). The difference was significant also for LPS 1000 ng/mL [3280 pg/mL (2709 to 4418) vs 2910 pg/mL (2313 to 3358); p=0.011] in which cell activation occurs non-specifically via low-affinity LPS receptors on monocytes (Heumann et al.,

1992). The basal TNF-α levels were low in both of the subject groups. The monocyte counts ($x10^9$/L) of the patients and controls were comparable [0.34 (0.29 to 0.46) vs 0.35 (0.28 to 0.44)].

The results showed that LPS-stimulated monocytes of persons with a history of AAU release TNF-α into the culture medium more than do monocytes of healthy subjects. The difference was significant in the presence of a low concentration of LPS (10 ng/ml) but also in the high, non-physiologic LPS concentration (1000 ng/ml).

The subgroup analysis of the data indicated that the TNF-α levels were similar in the HLA-B27-negative (n=7) and HLA-B27-positive patients (n=49) in the presence of LPS 10 ng/mL [1595 pg/mL (1130 to 2530) vs 1440 pg/mL (1205 to 2008); p=0.481]. In the presence of LPS 1000 ng/mL, the TNF-α levels were higher in the HLA-B27-negative patients [4445 pg/mL (3990 to 4785) vs 3190 pg/mL (2650 to 4178); p=0.022]. The TNF-α levels were similar for the HLA-B27 positive (n=8) and HLA-B27-negative controls (n=29) in the presence of LPS 10 ng/mL [1335 pg/mL (1031 to 1629) vs 1285 pg/mL (838 to 1555)] or 1000 ng/mL [2568 pg/mL (1900 to 3254) vs 3010 pg/mL (2438 to 3493)] and for the SpA group and non-SpA group (data not shown).

6.3.2 CD14 expression

In a comparison of the patients and controls, the proportions of $CD14^{bright}CD16^{-}$ monocytes [86.0% (82.3 to 88.1) vs 85.6% (82.8 to 89.0] and $CD14^{dim}CD16^{+}$ monocytes [4.5% (3.2 to 6.7) vs 4.3% (2.8 to 6.4)] were comparable. The median CD14 expression of the patients' $CD14^{bright}CD16^{-}$ monocytes [22 839 ABC (21038 to 26 020)] was similar to that of the $CD14^{bright}CD16^{-}$ control monocytes [21 657 ABC (19854 to 25646)].

6.3.3 CRP

The CRP concentration of the AAU patients was significantly higher than that of normal controls [1.59 μg/mL (0.63 to 3.47) vs 0.81 μg/mL (0.32 to 2.09); p=0.008]. The subgroup analysis showed that the CRP level of the patients with (n=12) and those without (n=44) SpA were comparable [(1.27 μg/mL (0.37 to 2.52) vs 1.68 μg/mL (0.81 to 3.60)].

6.3.4 sIL-2R

The sIL-2R levels of the patients' sera [334 IU/mL (267 to 417)] and control sera [394 IU/mL (284 to 478)] were comparable.

6.3.5 CD11b expression

The CD11b expression level of the patients' monocytes [92 RFU (87 to 104)] was similar to that of the control monocytes [86 RFU (80 to 100)].

7. DISCUSSION

7.1 HLA-B27 DISTRIBUTION, CLINICAL CHARACTERISTICS AND OUTCOME OF THE PATIENTS

Power and his colleagues (Power et al., 1998) showed that the prognosis of anterior uveitis associated with the HLA-B27 haplotype, either with or without associated systemic disease, was less favorable when compared with that of HLA-B27-negative patients with idiopathic anterior uveitis. In our series 5 out of 16 HLA-B27 negative AAU cases involved distinct uveitis entities when examined carefully. The remaining 11 did not differ in clinical manifestation from the idiopathic B27 positive ones, although the sample size was too small for definite conclusions. This phenomenon has been observed also previously by others (Linssen and Meenken, 1995). Further, by the time of the follow-up visit approximately one fourth of the patients suffered from eye complications, which did not associate with the presence of SpA or HLA-B27 antigen. Obviously, in addition to exogenous factors, it is likely that also other genetic factors besides HLA-B27 predispose patients to complicated course of the disease. These conclusions are supported by the results of epidemiological, family and twin studies (van der Linden et al., 1984b; Linssen et al., 1991; Järvinen, 1995).

Among our uveitis patients SpA was more common in men than women. This is in accordance with previous observations by others (Sampaio-Barros et al, 2001, Queiro et al., 2001). In addition to HLA-B27, hormonal factors may contribute to the manifestation of SpAs (James, 1991, Ostensen and Ostensen, 1998). Women appear to have more atypical SpAs than men and the systemic diseases are frequently undiagnosed before the onset of AAU and before referral to a rheumatologic examination by an ophthalmologist (Tay-Kearney, 1996).

At the first visit of the unilateral AAU patients in our study, only 13% were diagnosed as having some form of SpA, in contrast to the 24% to 90% reported earlier by other researchers (Stanworth and Sharp, 1956; Haarr, 1960; Mapstone and Woodrow, 1975; Russell et al., 1976; Pedersen, 1980; Saari et al., 1982; Vinje et al., 1983; Beckingsale et al., 1984; Wakefield et al., 1984; Feltkamp, 1985; Linssen et al., 1986; Rothova et al., 1987; Rosenbaum, 1989; Tay-Kearney et al., 1996; Power et al., 1998). Our series consisted strictly of patients with unilateral acute or recurrent anterior uveitis with no previous history of systemic diseases. In addition, 34 of the 72 patients with unilateral HLA-B27 positive AAU

had their first attack when entering our study, and they may not have had time to develop the symptoms characteristic of AS when examined at the first time. Consequently, in the follow-up visit, 25% of the unilateral AAU patients were diagnosed having SpA.

Based on our results and on previous studies, patients with typical symptoms of iritis and unilateral acute anterior eye involvement have an 80% probability of being HLA-B27 positive in Finland (Saari et al., 1984). Thus, one could argue that HLA-B27 typing is not necessary at all in such cases. However, this opinion can be challenged. First, in addition to having their prognosis estimated, AAU patients benefit from HLA-B27 typing because a positive test result alerts the clinician to the need to search for SpAs. Second, the lack of HLA-B27 antigen in a unilateral AAU patient may be a clue for the clinician to search for other specific uveitis entities and other systemic diseases.

The fact that HLA-B27 existed less often in patients with other uveitis entities than AAU (7.8% in tested) compared with the general population (14%) in Finland give credence to the idea of HLA-B27 having a protective role against other uveitis entities than AAU. It is possible that the genetic markers predisposing for example to posterior uveitis entities are not inherited in the same connection with the HLA-B27 allele. As follows, HLA-B27 antigen (Khan, 1995) and associated diseases are rare in non-Caucasian populations whereas posterior uveitis entities are more common (Sasaki et al., 1979, Biswas et al., 1996).

Our results suggest that the clinician does not benefit from HLA-B27 typing when uveitis is classified as bilateral and/or chronic or the inflammation is located in the posterior part of the eye. However, HLA-B27-positive AAU in connection with symptoms suspicious of SpA can sometimes develop into posterior or panuveitis. Indeed, a series of patients with seronegative arthritic syndromes and HLA-B27-associated uveitis with severe, sight-threatening, posterior segment ocular manifestations has been reported (Rodriguez et al., 1994). In accordance with this five patients out of the excluded group of 57 uveitis patients (Fig 1) were HLA-B27 positive and had also SpA. Their inflammation began as AAU but later on spread also into posterior uveal tissues. On the basis of these evidence HLA-B27 typing can be recommended in posterior or panuveitis when associated with symptoms of SpA. Interestingly, one of our male patients was HLA-B27 negative, but had had multiple recurrences of AAU and even macular edema once during the exacerbation of the eye disease. In addition, he had symptoms and findings fulfilling the diagnostic criteria for AS and UC. This finding is in line with other studies in which patients with AS and UC or Crohn's disease have been less often HLA-B27 positive than patients with AS alone (Dekker-Saeys et al., 1978, Palm et al., 2002). These different clinical and histocompatibility patterns suggest a mixed etiopathogenesis of AS in IBD patients. Weather it is a matter of greater bacterial antigen load leading to priming of polymorphonuclear neutrophils,

genetically determined over production of proinflammatory cytokines or inability to control inflammation cascade warrants further studies. Consequently, one must keep in mind that the absence of HLA-B27 antigen does not necessarily exclude the possibility of the patient having SpA, although the risk is considerably smaller.

7.2 INFECTIOUS BACKROUND AND SYSTEMIC INFLAMMATION

Taken together, elevated antibody levels could be a mark of subclinical infections in the mucosal membranes of the body (i.e. gut, urogenital region or respiratory tract) or sign of increased penetrance of microbial antigens. Indeed, increased fecal carriage of *Klebsiella* species (Ebringer et al., 1979), as well as increased serum IgA class antibody levels against *K. pneumoniae* and *E. coli* lipopolysaccharide (Mäki-Ikola et al., 1995) and higher frequency of IgG antibodies to *C. trachomatis* (van der Paardt et al., 2000) in association with AAU in patients with AS have been reported. Further, direct evidence for an abnormal humoral immune response as enhanced jejunal production of IgM, IgG, and IgA class antibodies to *K. pneumoniae*, and IgM and IgA class antibodies against *E. coli* and *P. mirabilis* in patients with AS compared with healthy controls has been found (Mäki-Ikola et al., 1997b). In theory it would have been beneficial to our study if all the patients with AAU would have had colonoscopy performed and/or measurement of gut immunity directly in jejunal fluid with the help of balloon perfusion device would have been performed. However, in clinical practice the recruitment of patients without symptoms of systemic inflammation for sometimes exhausting and painful examinations, not to mention the risk for complications is unethical. Although considerable skepticism can be raised on the role of elevated serum antibodies in the pathogenesis of AAU, our results are in line with afore mentioned theory and reports suggesting an indirect evidence of chronic inflammation and/or increased permeability at mucosa.

A single elevated antibody level may indicate that the patient may have been exposed to the microbe in the past. It may also derive from prolonged antigen persistence as observed in patients with SpA (Hoogkamp-Korstanje et al., 1988). In agreement with this, persisting antibody responses against bacteria occur in patients with ReA (Granfors et al., 1980, Mäki.Ikola et al., 1991). Furthermore, *Yersinia*, *Salmonella* and *Shigella* antigens or DNA have been shown to persist in synovial membrane (Hammer et al., 1990; Merilahti-Palo et al., 1991), synovial fluid (Granfors et al., 1989a; Granfors et al., 1990; Granfors et al, 1992) or peripheral blood cells (Granfors et al., 1998) in patients with ReA, but contradictionary findings have also been reported (Viitanen et al, 1991; Nikkari et al., 1992; Gaston et al., 1999; Nikkari et al., 1999; Wilkinson et al., 1999). Despite of the negative findings in the search

for antigenic material in PBMC in our study, it is intriguing to speculate its role in disease mechanism if detected. Indeed, it is not known how long *Yersinia* and *Salmonella* antigens persist in PBMCs. In our study serological markers of *Salmonella* and *Y. enterocolitica* infections were shown in 16% and 7.8-9.4% of patients respectively. Theoretically it is possible that persistence of these enterobacterial degradation products in the first place, even presently undetectable in the PBMCs, primed the host's immune system, which with following other bacterial infections might contribute to the outcome of these patients.

Reports of PCR positivity but seronegativity or antigen negativity have been published previously for both *C. pneumoniae* and viral carriers (Boman et al., 1998; Blasi et al., 1999; Taylor-Wiedeman et al., 1991). In accordance with that in one of our patients who was not positive for *C. pneumoniae* antibodies, *C. pneumoniae* DNA was detected. Given the high sensitivity of PCR, the presence of *C. pneumoniae* DNA in the absence of antibody response may simply reflect an early stage of the infection or very low levels of IgG antibodies. In contrast, failure to detect *C. pneumoniae* DNA in circulatory cells among seropositive patients may reflect very low levels of DNA, clearance of *C. pneumoniae* DNA from circulating cells over a period of time, or a reservoir of antigenic material, e.g. in lungs or in tissue macrophages. The latter possibility has been supported by the finding by Kaul et al who showed that two seropositive patients who initially tested positive for *C. pneumoniae* DNA were found to be PCR negative on repeated blood draw after an interval of five months. However, both patients continued to exhibit *C. pneumoniae* specific IgG antibodies (Kaul et al., 2000).

Our other finding showing more often and higher levels of IgA antibodies to Cpn Hsp60 in patients with AAU than in the controls may reflect a repeated or persistent infection in a host. Indeed, IgA antibodies to Cpn might play an important role in the defense mechanisms at the site of the mucosal surface in the respiratory tract. A key issue in chlamydial diseases is whether the pathologic mechanisms are associated with an enhanced immune response mediating tissue destruction through cytotoxic reactions (Ward, 1999), or whether they are related to the Th2 type of response that eventually leads to the partial or temporary suppression of an effective antichlamydial response (Th1 response) (Yang et al., 1996; Yang et al., 1999). In both models, chlamydial heat shock protein 60 (Hsp60) has been proposed to be the key antigen.

According to our results the prevalence and levels of IgA antibodies to *C. pneumoniae* were detected more often in controls than in patients. The utmost significance of IgA antibodies to *C. pneumoniae* has not been resolved yet. Among researches controversial opinions have been proposed. According to Saikku and co-workers (1988) IgA antibodies to *C. pneumoniae* are markers of chronic infection in patients with atherosclerosis. Comparably it is possible but not proven that our controls had more often

persisting infection caused by *C. pneumoniae* than our patients. One explanation could be that our controls have some subclinical chronic disease which is related to elevated levels of IgA antibodies to *C. pneumoniae* common in general population. On the other hand a remarkable percentage of our controls were hospital staff from the Oto-Rhino-Laryngological Department and they might have been predisposed to *C. pneumoniae* more often than our patients. Further, the serum samples of the patients were collected in the autumn 1999 and the samples of the controls in January and February 2000 although there were no epidemic of *C. pneumoniae* during that time period.

Interestingly, our findings showed more often and higher levels of IgA antibodies to Cpn Hsp60 in patients with AAU than in the controls in contrast to IgA antibodies to *C. pneumoniae*. First, antibodies to *C. pneumoniae* detectable by MIF and antibodies against Hsp60 do not necessarily correlate with each other. In MIF the outer surface of the purified particles of *C. pneumoniae* serves as a antigen. MIF detects antibodies produced against the outer surface of *C. pneumoniae* bacteria. Hsp60 is considered to be a cytoplasmic protein. Cpn Hsp60 gene has been cloned into Bacillus subtilis bacteria capable of producing CpnHsp60 protein (Hsp60). The protein is purified and used as antigen in EIA. Hsp60-EIA detects then antibodies produced against CpnHsp60. Second, cross reactivity between host proteins and chlamydial Hsp60 is possible. In EIA procedure antibody response is measured with proteins that could be denaturated and the antibodies against epitopes present in native proteins may remain undetected.

In our study markers of uveitis activity and the development of SpA did not correlate with titer of IgA antibodies to Cpn Hsp60. This result is in a agreement with the findings of Wakefield et al (1983) who showed that AU patients with and without associated rheumatic disease do not differ in respect to a cell-mediated response to chlamydial genus specific antigen or antibody response. In contrast, we observed that ocular complications were more frequent in patients with high levels of IgA antibodies to CpnHsp60 than in patients with lower IgA responses. These results are in line with previous published reports suggesting that the production of chlamydial Hsp60 is increased in cases of persistent infection and that chlamydial Hsp60 can be a causal factor in the immunopathogenesis of various complications induced by persistent infection (Wagar et al., 1990, Toye et al., 1993, Peeling et al., 1997, Eckert et al., 1997, Money et al., 1997). Indeed, the association of IgA responses of the patients with the worst ocular manifestations may reflect greater loads of persistent infection at mucosal surfaces such as the lung.

Another explanation for the prevalence of antibodies observed in patients with recurrent or complicated course of the disease is provided by modified microbial invasion and/or altered elimination of microbial components. Results of experimental studies suggest a link between HLA-B27 and Gram-negative bacteria. Consequently, Kapasi and Inman (1992) showed a diminished invasion of Gram-

negative bacteria into HLA-B27-transfected mouse fibroblasts, an inverse relationship between invasion and the expression of HLA-B27. However, later studies with opposite results have been presented (Hupperts and Heesemann, 1996). Interestingly, rats and mice transgenic for HLA-B27 have shown enhanced susceptibility to infection with *Y. enterocolitica* (Nickerson et al., 1990) and *Listeria monocytogenes* (Warner et al., 1996) suggesting that HLA-B27 may contribute to defective immunity against microbes. In contrast, in the bacterially induced AAU (Baggia et al., 1997) the expression of HLA-B27 did not appear to influence the incidence or severity of uveitis in B27+ low-copy heterozygous rats. Finally, HLA-B27 has been demonstrated to interfere with antigen elimination in *Salmonella* infected human monocytic U937 cells (Ekman et al., 1999). Although the higher persistence and/or antibody levels in the present study was observed in AAU patients irrespectively of the HLA-B27 status, it is possible that the statistical significance was not reached because of a low number of HLA-B27 negative patients and HLA-B27 positive healthy controls. This warrants further investigation with groups of patients and controls matched with equal amount of HLA-B27 positivity and negativity to reach the statistical power.

High innate immune responsiveness could explain the elevated antibody levels observed by us and our results showing that LPS-stimulated monocytes of persons with a history of AAU release TNF-α into the culture medium more than do monocytes of healthy subjects. The difference was significant in the presence of a low concentration of LPS (10 ng/ml) and also in the high, non-physiologic LPS concentration (1000 ng/ml). These findings suggest that hyperresponsiveness of the patients' monocytes is not confined to the high-affinity CD14 receptor pathway but may also involve other cell surface receptors and their intracellullar signaling pathways. In experimental EIU, and most probably in patients with AAU, mononuclear phagocytes are recruited into the anterior uvea (Cousins et al., 1984). Upon emigration into the eye, monocytes become activated. If hyperreactive in terms of TNF-α production, they may be capable of breaking down innate immune privilege in the eye.

One mechanism for enhancing monocyte TNF-α production in response to low levels of LPS could be priming of monocytes by LPS and humoral mediators of inflammation (Kitagawa et al., 1996). An enhanced permeability of the gut in patients with AS has been reported (Leirisalo-Repo et al., 1995, Mielants et al., 1995). This could allow LPS to pass through mucosa into circulation and prime monocytes. In our study, however, the TNF-α levels in the culture media of SpA monocytes were similar to those of non-SpA monocytes. This finding may reflect the fact that our patients had milder and relatively shorter course of the SpA compared with subjects reported by other studies (Callahan and Pincus 1995; Mäki-Ikola et al., 1997b).

It is possible that the HLA-B27 gene itself may play a modulatory role in the activation of the innate immune system. Ikawa et al (1998) have reported that the expression of HLA-B27 on Hela cells promotes induction of c-*fos* in response to *in vitro* invasion by *S. typhimurium*, indicating that HLA-B27 may be associated with the activation of otherwise silent intracellular signal transduction pathways leading to the activation of innate immune genes. This finding is in concordance with our results of enhanced TNF-α production by LPS-stimulated whole blood obtained from persons with previous AAU, most of who were positive for the HLA-B27 antigen. The limited number of HLA-B27-negative persons in the AAU group (n=7) tended to show even higher TNF-α production than did the HLA-B27-positive subjects. This tendency suggests that enhanced TNF-α production may play a role in the pathogenesis of AAU in the HLA-B27-negative patients as well. On the other hand, the low number of healthy control subjects who were positive for HLA-B27 (n=8) had TNF-α production similar to that of the HLA-B27-negative control subjects. This finding indicates that the presence of HLA-B27 gene is not consistently associated with enhanced TNF-α production when it occurs in patients with previous AAU. If confirmed in person groups with sufficient statistical power, our findings suggest that enhanced TNF-α production is associated with previous AAU but not with each of the 23 HLA-B27 alleles identified so far (Ball and Khan, 2001). In this context it is of interest that there are differences in susceptibility to AAU between various subtypes of HLA-B27. Indeed, B*2704 seems to be less susceptible to AU compared with B*2705 in Japanese subjects (Konno et al., 1999). Further, B*2706 in Indonesia and B*2709 in Sardinia are not associated with SpA (Feltkamp et al., 2001). The possibility that such differences derive from the TNF-α production capacity warrants further studies.

In our study the proportions of $CD14^{bright}CD16^{-}$ monocytes and $CD14^{dim}CD16^{+}$ monocytes and the median CD14 expression of the $CD14^{bright}CD16^{-}$ monocytes were similar between patients and controls. $CD14^{dim}CD16^{+}$ monocytes are associated with increased TNF-α production (Frankenberger et al., 1996); yet, their proportion was not increased in our study. A genetic factor may affect innate immune responsiveness. The TNF-α gene shows promoter region polymorphism, and the TNF-2 allele has been associated with high, inducible levels of TNF-α (Wilson et al., 1997), but not in all studies (Brinkman et al., 1996; Stuber et al., 1996). The promoter region polymorphism of the CD14 gene is associated with high monocyte CD14 expression (Meisel et al.,m 1998; Hubacek et al., 1999; Shimada et al., 2000). In our study, the differences in the CD14 expression level between the patients and controls were not significant; this finding suggests that CD14 density may not explain the difference in LPS responsiveness.

CRP serves as a marker of systemic inflammation in acutely ill patients in whom a systemic inflammatory reaction has been triggered by a variety of infectious and non-infectious insults (Takala et al., 1999a; Takala et al., 1999b; Takala et al., 2000). Although CRP levels in patients with AAU are

not so infrequently elevated (Sprenkels, 1995) most of these patients also have SpA. Because the subjects included in our study did not have any marks of acute inflammation, we expected that the CRP levels would be low, and we therefore used the high-sensitivity CRP assay. To our knowledge, such an assay has not been used previously in studies of patients with AAU or previous AAU. In apparently healthy young adults, the median value for CRP was 0.8 μg/mL, increased with age, and tended to be higher in women (Hutchinson et al., 2000). In our study, the CRP levels were significantly higher in the patients than in the controls. The finding cannot be explained by the differences in age or gender between the patients and controls. The elevated CRP levels in the patients may derive from increased intestinal permeability, which would permit the leakage of LPS from the gut into the circulation. This mechanism may operate in patients with chronic inflammatory bowel disease, known to be associated with SpA (Leirisalo-Repo et al., 1994). In our study, however, the CRP levels of the SpA patients were lower, although not significantly lower, than those of the non-SpA patients. Finally, LPS occurs in healthy subjects at concentrations of up to 20 pg/ml of peripheral blood (Opal et al., 1999) and up to 1 ng/ml of portal venous blood (Knolle et al., 1999). It is possible, but not proven, that patients develop stronger responses to physiologic concentrations of LPS than controls.

The levels of sIL-2R are elevated in patients with systemic inflammation triggered by a variety of infectious and non-infectious disorders (Takala et al., 1999a; Takala et al., 1999b; Takala et al., 2000). They are elevated also in patients during the acute phase of AU (Scheinberg et al., 1992; Martin et al., 2000), but not in patients who have recovered from AAU, as shown by our results. The innate and adaptive immune mechanisms are closely linked to each other (Palucka and Banchereau, 1999) and the former directs the development of the latter (Fearon, 1999).

Taken together, our findings suggest that the enhanced immune responsiveness observed in the persons with previous AAU was confined to an innate response (TNF-α production) and did not involve an adaptive response (T-cell proliferation).

Enhanced CD11b expression provides a useful means for evaluating phagocyte activation as a sign of systemic inflammation (Repo et al., 1997; Repo and Harlan, 1999; Takala et al., 1999a; Takala et al., 1999b; Vuorte et al., 1999; Takala et al., 2000). In our study, the monocyte CD11b expression levels were similar in the patients and controls. Although the finding suggests that the patients' phagocytes were not activated, the possibility remains that they were primed. Indeed, the phagocytes which express CD11b at normal, constitutive levels may be primed and show increased capacity of effector cell functions, such as the release of toxic reactive oxygen intermediates (Condliffe et al., 1996).

Regarding the pathogenesis of AAU, antibiotic treatment trials have failed to show that antibiotics would be superior to placebo (Wakefield et al., 1999). On the other hand, antibiotic treatment for 3 months has been shown to be beneficial for patients with *Chlamydia*- but not for with enterobacteria-induced ReA (Lauhio et al., 1991, Toivanen, 2001). Further, on the basis of the evidence one might suspect that anti-TNF-α treatment would be beneficial for patients with AAU. Paradoxically, treatment trials have questioned the pro-inflammatory effect of TNF-α in EIU. In three separate rodent studies using different anti-TNF-α administered both systemically and locally, disease was either not influenced or ocular inflammation was exacerbated (Rosenbaum and Boney, 1993; Kasner et al., 1993; De Vos et al., 1995). Data relating to antibody therapies are difficult to interpret. It cannot be certain that the agent has reached the required site at the appropriate time in sufficient quantities. However, it has been shown that TNF-receptor p55 gene knock-out mice suffer significantly less intra-ocular inflammation than normal phenotype controls, and that mice deficient in both TNF-receptor p55 and TNF-receptor p75 show less from intra-ocular inflammation than mice deficient in TNF-receptor p75 alone (Smith et al., 1998b). These results imply that TNF-α does have a pro-inflammatory influence in EIU and most probably in AAU. Indeed, anti-TNF-α therapy has been observed to be highly effective in patients with AS (Brandt et al., 2000, Gorman et al., 2002) and beneficial to certain subgroups of patients with AAU, but more effective in controlling associated SpA (Smith et al., 2001).

In conclusion, the pathogenesis of AAU comprises triggering of inflammation, amplification of the process, and development of inflammatory tissue injury. Extensive exposure to or impaired elimination of microbial components may result in an antigen over-load in the body, triggering an inflammatory cascade. Further, inadequate elimination leads to persistence of microbes and enhanced phagocyte and complement activation in addition to exaggerated production of proinflammatory cytokines, oxygen radicals and lysosomal enzymes, which in turn cause tissue injury and amplify the vicious circle of inflammation. HLA-B27 has been suggested to interfere with the invasion and modulate the intracellular survival of bacteria suggested to trigger AAU. In our study HLA-B27 antigen was highly prevalent among patients with unilateral AAU in contrast to uveitis entities affecting the posterior part of the eye or bilateral or chronic cases. Although HLA-B27 positivity did not appear to affect the biomicroscopic features or the prognosis of the patients with AAU, these findings do not rule out afore mentioned theories. Indeed, elevated antibody responses observed in our patients with a high frequency of HLA-B27 and with many recurrences of AAU, compared with patients with none or few recurrences, could be a sign of repeated infections, antigen persistence due to impaired elimination and/or elevated production of proinflammatory cytokines as a marker of enhanced innate immune responsiveness. Furthermore, the high frequency of antibodies to Cpn Hsp60 observed in patients with a history of AAU could indicate that the patients have persisting or recurrent infections due to *C.*

pneumoniae. Our finding suggests that *C. pneumoniae* may play a role in the pathogenesis of AAU and result in a complicated outcome.

Last, the theory of enhanced innate immune responsiveness gained support by our findings that in comparison with healthy subjects, the patients with a history of AAU had elevated levels of circulating CRP, and their monocytes generated higher levels of TNF-α *ex vivo* in response to LPS. Indeed, increased inflammatory reactivity may render the subjects susceptible to ocular inflammation by overcoming the mechanisms that maintain innate immune privilege in the eye.

7.3 METHODOLOGICAL POINTS

7.3.1 Antibodies to *Chlamydia pneumoniae* and human heat shock protein

On the basis of the evidence, one could argue that antibodies to chlamydial Hsp60 can represent a marker for autoimmune responses to self-Hsp60 (Oldstone, 1987). Antibodies to chlamydial Hsp60 cross-react with peptide epitopes from human Hsp60 (Yi and Brunham, 1993). It has been proposed that antibodies that have developed to Hsp during bacterial infection or T lymphocytes activated by Hsp can trigger an autoimmune reaction through molecular mimicry of host cells (Winfield and Jarjour, 1991). Especially in intracellular bacterial infections the pathogen enters a hostile environment and increases the regulation of its Hsp production (Peeling et al., 1999). In our study, however, the levels of IgA antibodies to human Hsp60 were low in both the patients and controls. This finding suggests that the marked levels of IgA antibodies to Cpn Hsp60 were a real indicator of ongoing immune reaction caused by *C. pneumoniae* infection.

The question also arises of whether our assay distinguishes Cpn Hsp60 from *C. trachomatis* Hsp60. Both Hsp60s are partly homologous. Thus an infection by either *C. pneumoniae* or C. *trachomatis* would induce an antibody response to shared antigens of these agents, including an antibody response to heat shock proteins. Because our patients did not have any marked serological evidence of previous *C. trachomatis* infection, but indeed evidence of *C. pneumoniae* infection, we reasoned that also the antibody response to Cpn Hsp60 would be specific to *C.pneumoniae* infection.

7.3.2 Production of TNF-α

Because monocyte purification procedures cause cell loss and changes in monocyte function, which may be misleading (Desch et al., 1989; Repo et al., 1991), we employed a whole blood setting tailored to evaluate the responsiveness of monocytes *in vivo*. The crucial question is whether monocyte TNF-α production by LPS-stimulated whole blood represents monocyte activation and innate immune responsiveness of the host. Several pieces of evidence suggest that this is the case. Although different types of leukocytes can produce TNF-α in given assay conditions, monocytes are considered to be the major source of TNF-α in the LPS-stimulated whole blood assay (Desch et al., 1989). In agreement, we found a positive correlation between the count of CD14-positive monocytes and the TNF-α level in the culture supernatant. The near maximal concentrations of TNF-α are obtained within the first four hours of incubation (Desch et al., 1989; Wilson et al., 1991, Nerad et al., 1992). In contrast to monocytes, neutrophils, which are capable of producing TNF-α during an 18-hour culture period (Bazzoni et al., 1991), and lymphocytes remain mainly negative for TNF-α during the early hours of LPS stimulation in whole blood (Heagy et al., 2000). Finally, the whole blood assay seems to provide a rapid means for evaluating innate responses to microbial structures in patients (Heumann et al., 1998) and in experimental settings in healthy subjects (Jörgensen et al., 2001).

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